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(54) Title: PREPARATION AND METHOD FOR PREVENTION OF COCCIDIOSIS

(57) Abstract: Preparations and methods are disclosed for the vaccination of members of the class *Aves* against coccidiosis by intra yolk sac injection of sporocysts or sporulated oocysts treated to disrupt the oocyst wall.

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PREPARATION AND METHOD FOR PREVENTION OF COCCIDIOSIS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority from provisional application Serial No. 60/163,898, filed November 8, 1999, which is hereby incorporated by reference in its entirety for all purposes.

BACKGROUND

Coccidiosis is a disease of various animals in which the intestinal mucosa is invaded and damaged by a protozoa of the subclass *Coccidia*. The economic effects of coccidiosis can be especially severe in the poultry industry where intensive housing of birds favors the spread of the disease. Infection by coccidial protozoa is, for the most part, species specific. Numerous species, however, can infect a single host. For example, there are seven species of *Coccidia* which infect chickens, six of which are considered to be moderately to severely pathogenic.

The life cycle of the coccidial parasite is complex. For example, protozoa of the genera *Eimeria*, *Isospora*, *Cystoisospora*, or *Cryptosporidium* typically only require a single host to complete their life cycle, although *Cystoisospora* may utilize an intermediate host. Under natural conditions, the life cycle begins with the ingestion of sporulated oocysts from the environment. When sporulated oocysts are ingested by a susceptible animal, the wall of the sporulated oocyst is broken in order to release the sporocysts inside. In poultry, the release of the sporocyst is the result of mechanical disruption of the sporulated oocyst in the gizzard. Within the sporocysts, are the sporozoites which are the infective stage of the organism. In poultry, the breakdown of the sporocyst coat and release of the sporozoites is accomplished biochemically through the action of chymotrypsin and bile salts in the small intestine. Once released, the sporozoites invade the intestinal mucosa or epithelial cells in other locations. The site of infection is characteristic of the species involved. For example, in the genus *Eimeria*, *E. tenella* is localized in the ceca; *E. necatrix* is found in the anterior and middle portions of the small intestine; *E. acervulina* and *E. praecox* occur in the upper half of the small intestine; *E. brunetti* occurs in the lower small intestine, rectum, ceca, and cloaca; while *E. mitis* is found in the lower small intestine.

Once inside the host animal's cells, sporozoites develop into multinucleate meronts, also called schizonts. Each nucleus of the meront develops into an infective body called a merozoite which enters new cells and repeats the process. After a variable number of asexual generations, merozoites develop into either microgametocytes or
5 macrogametes. Microgametocytes develop into many microgametes which, in turn, fertilize the macrogametes. A resistant coat then forms around the resulting zygotes. The encysted zygotes are called oocysts and are shed unsporulated in the feces. Infected birds may shed oocysts in the feces for days or weeks. Under proper conditions of temperature and moisture, the oocysts become infective through the process of sporulation.
10 Susceptible birds then ingest the sporulated oocysts through normal pecking activities or ground/litter foraging and the cycle repeats itself. Ingestion of viable, sporulated oocysts is the only natural means of infection.

Infection with coccidial protozoa results in immunity so that the incidence of the disease decreases over time as members of the flock become immune. This self-limiting
15 nature of coccidial infections is widely known in chickens and other poultry. The immunity conferred, however, is species specific such that introduction of another species of coccidial protozoa will result in a new disease outbreak.

The oocyst wall of coccidial protozoa provides a highly effective barrier for oocyst survival. Oocysts may survive for many weeks outside the host. In the laboratory, intact
20 oocysts are resistant to extremes in pH, to detergents, to proteolytic, glycolytic, and lipolytic enzymes, to mechanical disruption, and to chemicals such as sodium hypochlorite and dichromate.

Both chemotherapy and vaccination are currently used to control coccidiosis in poultry. Numerous drugs are available for the control of coccidiosis in poultry. Because of
25 the number of species which cause the disease, very few drugs are efficacious against all species, although a single drug may be efficacious against several species. In modern broiler chicken production, for example, administration of drugs to control coccidiosis is routine and represents a significant cost of production.

Two programs of drug administration are commonly used in the domestic poultry
30 industry. The simplest is the continuous use of a single drug from day one until slaughter. The shuttle or dual drug program involves the use of two different drugs, one administered in the "starter" ration and a second drug administered in the "grower" ration. The second

method is often preferred as a method to minimize development of drug resistant strains of coccidial protozoa. Using either method, drugs used are typically rotated two to three times per year in order to minimize the development of resistant strains.

5 The development of drug resistance by coccidial protozoa is a serious limitation on the effectiveness of chemotherapy to control the disease. Surveys in the United States, South America and Europe have revealed widespread drug resistance. Since drug resistance is a genetic phenomenon, once established, drug resistance can remain in the population for many years until reduced by natural selection pressure and genetic drift.

10 The use of drugs in animals used for food production is also coming under increasing scrutiny by the public. Consumers are increasingly concerned with the possibility of drug residues in food. This creates pressure in the poultry industry to reduce the use of drugs to control coccidiosis.

15 Vaccination of birds against coccidiosis is an alternative to chemotherapy. An advantage of vaccination is that it can greatly reduce or eliminate the need to administer anti coccidial drugs, thus reducing drug costs to poultry producers, preventing the development of drug-resistant strains, and lessening consumer concerns about drug residues.

20 Numerous methods have been developed to immunize poultry against coccidiosis. The successful methods have all been based on the administration of live protozoa, either fully virulent strains or attenuated strains. The most common route of administration is oral, although other routes have been used. Edgar, U.S. Patent No. 3,147,186, teaches vaccination of chickens by oral administration either directly into the mouth or via the feed or water of viable *E. tenella* sporulated oocysts. Davis et al., U.S. Patent No. 4,544,548, teaches a method of vaccination by continuous administration of low numbers of sporulated oocysts, with or without simultaneous administration of anti coccidial drugs.

25 Oral administration of attenuated strains of sporocysts has also been utilized to confer immunity against coccidiosis (Shirley, U.S. Patent No. 4,438,097; McDonald, U.S. Patent No. 5,055,292; and Schmatz et al., PCT publication No. WO 94/16725). An alternative to attenuation is disclosed in Jenkins et al., *Avian Dis.*, 37(1):74-82 (1993), which teaches the oral administration of sporozoites that have been treated with gamma radiation to prevent merogonic development.

The use of oral vaccines for the prevention of coccidiosis in poultry, however, has several undesirable characteristics, especially when applied to commercial broiler operations. When the vaccine is administered in the feed and/or water, dosage uniformity can be a problem since the amount of vaccine received is controlled, not by the producer, but by the amount of feed or water the birds consume. If the vaccine is given per os, there is a risk of an overdose which may result in a disease outbreak if fully virulent forms of coccidial protozoa are used. Oral vaccination with virulent forms of coccidial protozoa has also been associated with declines in gain and feed conversion resulting from sub-clinical infections. In addition, oral vaccination results in substantial shedding of coccidial oocysts by vaccinated birds. Shedding occurs because oral vaccines work by establishing a sub-clinical infection in the digestive tract resulting in the production of oocysts which are then shed in the excreta. This shedding results in a build up of oocysts in the litter presenting an increasing environmental challenge which can result in disease outbreaks when virulent forms are used.

Parenteral routes of vaccination have included subcutaneous or intraperitoneal injection of excysted sporozoites, (Bhogal, U.S. Patent No. 4,808,404; Bhogal et al., U.S. Patent No. 5,068,104), and intra ovo injection of either oocysts or sporocysts, (Evans et al., PCT publication No. WO 96/40233; Watkins et al., *Poul. Sci.*, 74(10):1597-602 (1995)). Sharma, *J. Parasitol.*, 50(4):509-517 (1964), reported unsuccessful immunization trials involving intravenous, intraperitoneal, intramuscular, or subcutaneous injection of either viable oocysts or a mixture of oocysts, sporocysts and sporozoites. Thaxton, U.S. Patent No. 5,311,841, teaches a method of vaccination against coccidiosis by administration of oocysts or sporozoites to newly hatched chicks by yolk sac injection.

Vaccination against coccidiosis by injection has advantages over oral vaccination. Injection results in greater uniformity of dosage, since it is the operator and not the bird who controls the amount of vaccine received. Vaccination by injection also results in substantially less shedding of coccidial organisms by vaccinated birds and so causes less environmental build up. Vaccination by injection, however, requires a large input of labor with the currently available injection equipment. Intravenous and subcutaneous vaccination routes require precise placement of the injection needle which does not lend itself to mass vaccination. Due to the small size of young chicks, there is an increased risk of injury with intramuscular injection due to the injection needle missing the muscle and

hitting either bone or nervous tissue. The small size of the muscles in newly hatched chicks also greatly limits the volume of material that can be injected without causing damage to the muscle. In addition, if not done properly, intramuscular injection can result in the formation of injection cysts in the muscle which lessens the value of the carcass.

5 Intra yolk sac injection provides an advantageous route for the administration of various medications and vaccines, including coccidiosis vaccines. The yolk sac is a membrane-bound, extraembryonic structure attached to the midgut of the developing embryo by the yolk stalk, which stores nutrients, in the form of yolk, that are utilized by the embryo during development. In addition, the yolk can serve as a source of maternal
10 antibodies to the developing embryo. Just before hatching, the yolk sac is internalized and delivers its contents over time to both the blood stream via blood vessels in the yolk sac membrane, and into the intestinal lumen. During the first three to seven days post-hatching, the yolk sac membrane actively and passively transports intact macromolecules to the bloodstream of the newly hatched chick. In addition, during the first few days after
15 hatching, the yolk sac delivers the residual yolk into the intestinal lumen via the yolk stalk. Thus, material placed in the yolk sac, including coccidial protozoa, will be transported over time into the intestine. Also during the first few days after hatching, the yolk sac membrane is invaded by lymphoid tissue, so that any organism placed in the yolk sac will be exposed to the lymphatic system.

20 Although intra yolk sac vaccination for coccidiosis would appear to have several advantages, its wide-spread use has been hampered by the lack of a coccidiosis vaccine preparation suitable for intra yolk sac administration. Traditional oral coccidiosis vaccines contain oocyst stage coccidial protozoa. As discussed previously, oocysts are surrounded by a protective coat which is normally removed by mechanical action in the gizzard.
25 When delivered via the yolk sac, the gizzard is bypassed so that the oocyst coat is not removed. Without being limited by theory, the requirement of live protozoa to confer immunity suggests that the infective sporozoite form of the protozoa must be released to elicit an immune response. Thus, it would appear that oocysts, or any other encysted form of the protozoa, would have limited effectiveness as an intra yolk sac vaccine. Excysted
30 forms of coccidial protozoa, such as sporozoites, can be used for intra yolk sac vaccination against coccidiosis (Thaxton, U.S. Patent No. 5,311,841). Excysted forms of coccidial protozoa have a disadvantage, however, in that they are more difficult to maintain in a

viable state *in vitro* as compared to encysted forms. In addition, coccidial vaccines for injection must be of a higher purity than vaccine preparations administered per os. For example, and without limitation, minor bacterial, viral or fungal contamination of an oral vaccine preparation will have little, if any, effect on the animal to which it is administered.

- 5 In contrast, even minor bacterial, viral or fungal contamination of an injected preparation can result in substantial harm. Likewise, compounds used in the vaccine preparation which are safe when taken by mouth, may be harmful when injected into the body.

SUMMARY

- 10 Among the several aspects of the invention, therefore, is to provide a preparation for the prevention and treatment of coccidiosis in members of the class *Aves* by intra yolk sac administration of the preparation into newly hatched chicks. The preparation comprises, live sporocysts of a coccidial protozoa in a carrier suitable for injection and is substantially free of extraneous bacterial, fungal and viral contaminants.

- 15 One aspect provides a preparation for administration by intra yolk sac injection for the prevention and treatment of coccidiosis in members of the class *Aves* comprising, live sporocysts of at least one species of coccidial protozoa and pharmaceutically acceptable carrier, diluent or excipient, the preparation characterized by being substantially free of extraneous bacterial, fungal and viral contaminants.

- 20 Another aspect provides a preparation for administration by intra yolk sac injection for the prevention and treatment of coccidiosis in members of the class *Aves* comprising, live sporulated oocysts of at least one species of coccidial protozoa which have been treated to disrupt the oocyst wall and pharmaceutically acceptable carrier, diluent or excipient, the preparation characterized by being substantially free of extraneous bacterial, fungal and viral contaminants.

- 25 An additional aspect provides a preparation for administration by intra yolk sac injection for the prevention and treatment of coccidiosis in members of the class *Aves* comprising at least about 50 live, sporulated oocysts which have been treated to disrupt the oocyst wall from each of the species of coccidial protozoa *E. acervulina*, *E. maxima* and *E. tenella* and a pharmaceutically acceptable adjuvant, carrier, diluent, or excipient, the
30 preparation characterized by being substantially free of extraneous bacterial, fungal and viral contaminants.

Still another aspect provides a preparation for administration by intra yolk sac injection for the prevention and treatment of coccidiosis in members of the class *Aves* comprising at least about 50 live sporocysts from each of the coccidial protozoa *E. acervulina*, *E. maxima* and *E. tenella* and a pharmaceutically acceptable adjuvant, carrier, diluent or excipient, the preparation characterized by being substantially free of extraneous bacterial, fungal and viral contaminants.

Yet another aspect provides method for the prevention and treatment of coccidiosis in members of the class *Aves* comprising administration by intra yolk sac injection to newly hatched chicks of a preparation comprising live, sporocysts of at least one species of coccidial protozoa.

A further aspect provides a method for the prevention and treatment of coccidiosis in members of the class *Aves* comprising administration by intra yolk sac injection to newly hatched chicks of a preparation comprising live, sporulated oocysts of at least one species of coccidial protozoa which have been treated to disrupt the oocyst wall.

Another aspect provides method for the prevention and treatment of coccidiosis in members of the class *Aves* comprising administration by intra yolk sac injection to newly hatched chicks of a preparation comprising at least about 50 live, sporulated oocysts of each of the coccidial protozoa *E. acervulina*, *E. maxima* and *E. tenella* which have been treated to disrupt the oocyst wall.

Yet another aspect provides a method for the prevention and treatment of coccidiosis in members of the class *Aves* comprising administration by intra yolk sac injection to newly hatched chicks of a preparation comprising at least about 50 live, sporocysts of each of the coccidial protozoa *E. acervulina*, *E. maxima* and *E. tenella*.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with regard to the following description, appended claims, and accompanying drawings where:

Fig. 1 shows the effect of dose of treated *E. tenella* oocysts on lesion scores following oral challenge with *E. tenella*

Fig. 2 shows the effect of vaccination against *E. tenella* by intra yolk sac injection of either intact or treated oocysts on feed efficiency as measured by feed to gain ratios following oral challenge with *E. tenella*.

5 Fig. 3 shows the effect of vaccination against *E. tenella* by intra yolk sac injection of either intact or treated oocysts on body weight gain following oral challenge with *E. tenella*.

Fig. 4 shows the effect of vaccination against *E. maxima* by intra yolk sac injection of either intact or treated oocysts on lesion scores following oral challenge with *E. maxima*.

10 Fig. 5 shows the effect of vaccination against *E. maxima* by intra yolk sac injection of either intact or treated oocysts on feed efficiency as measured by feed to gain ratios following oral challenge with *E. maxima*.

15 Fig. 6 shows the effect of vaccination against *E. maxima* by intra yolk sac injection of either intact or treated oocysts on body weight gain following oral challenge with *E. maxima*.

Fig. 7 shows the number of oocysts shed in the excreta of birds administered 250,000 oocysts by either oral gavage or intra yolk sac injection.

20 Fig. 8 shows the effect of vaccination against *E. tenella* by intra yolk sac injection with treated oocysts, oral vaccination or administration of anti coccidial drugs on lesion scores of birds housed in floor pens.

Fig. 9 shows the effect of vaccination against *E. tenella* by intra yolk sac injection with treated oocysts, oral vaccination or administration of anti coccidial drugs on post challenge weight gain of birds housed in floor pens.

25 Fig. 10 shows the effect of vaccination against *E. tenella* by intra yolk sac injection with treated oocysts, oral vaccination or administration of anti coccidial drugs on post challenge feed to gain ratios of birds housed in floor pens.

Fig. 11 shows the effect on post challenge weight gain of vaccination against *E. tenella* with or without concurrent administration of an anti coccidial drug in the feed.

DEFINITIONS

30 "Chick" refers to the offspring of any member of the class *Aves*.

“Effective dose”, unless indicated otherwise, refers to the number of sporocysts or treated oocysts of a single coccidial species or a combination of coccidial species sufficient to confer immunological protection that is greater than the inherent immunity of non-vaccinated birds.

5 “Encysted protozoa”, “encysted oocyst” and “encysted sporocyst” all refer to organisms which are within a cyst or have their own outer coat or shell.

 “Excysted protozoa” and “excysted sporozoite” both refer to an organism in which the outer shell or coat has been removed either naturally or through artificial means.

 The term “extraneous” when used in reference to bacterial, viral or fungal
10 contamination refers to the presence of a bacterium, virus or fungus other than a bacterium, virus or fungus intentionally added to and intended to be present in the final form of the present invention. For example, in a preparation that also contained a viral vaccine, the presence of the virus added would not be extraneous viral contamination.

 “Final container” refers to any container containing at least one dose of the
15 preparation of the present invention that has undergone all processing steps except for testing for bacterial, viral or fungal contamination.

 “Newly hatched” refers to the time period within 72 hours after hatching.

 “Oocyst” refers to the dormant life-cycle stage of a coccidial protozoan having a tough outer coat. As formed, the oocyst is not capable of infection and may also be
20 referred to as an unsporulated oocyst. Oocysts are found in the intestine of animals following release from infected cells and are eliminated in the excreta.

 “Sporocyst” refers to a life-cycle stage of a coccidial protozoan having an outer coat or case containing a multiplicity of sporozoites which are the ultimate infective agent of the protozoan. In the instance of *E. tenella*, each sporocyst contains two sporozoites.

25 “Sporulated oocyst” refers to an oocyst which has undergone maturation naturally or through artificial manipulation such that the sporulated oocyst is capable of infecting a susceptible host. During maturation, a multiplicity of sporocysts, each with its own outer shell or case, develops within the oocyst creating a “cyst within a cyst.” For example, in *E. tenella*, a sporulated oocyst contains four sporocysts, each with their own outer shell or
30 case.

 “TCID” means tissue culture infective dose.

“Treated oocyst” refers to a sporulated oocyst that has been treated by any suitable means to disrupt the oocyst coat, thus allowing for the possible escape of the sporocyst within the sporulated oocyst.

“Trickle vaccination” or the trickle method is a method of vaccination against coccidiosis in which birds are continuously administered low numbers of coccidial parasites through the feed or water over more than one day.

“PBS” means phosphate buffered saline.

DETAILED DESCRIPTION

All publications, databases, patents and patent applications cited in the application are herein incorporated by reference in their entirety as if each individual publication, patent or patent application were specifically and individually indicated to be incorporated by reference.

Surprisingly, it has been discovered that preparations of sporocysts, or of sporulated oocysts which have been treated to disrupt the oocyst wall and thus allow release of the sporocysts inside, effectively confer immunity when administered by intra yolk sac injection. Preparations containing sporocysts or treated oocysts are superior to preparations containing untreated oocysts because they require substantially fewer numbers of oocysts in order to confer immunity. Sporocysts or treated oocysts are also superior to preparations containing primarily excysted stages, such as sporozoites. Sporozoites and other forms of excysted coccidial protozoa, are very fragile and lose their effectiveness to confer immunity in a few days unless considerable effort is made to maintain their viability. In contrast, sporocysts will maintain their viability and ability to confer immunity for long periods of time under relatively simple conditions, such as in phosphate buffered saline (PBS). More importantly, the present invention has been shown to be as effective as the traditional methods for prevention of coccidiosis, namely oral vaccination and chemotherapy by administration of anti coccidial agents in the feed and water, when tested under conditions replicating those encountered in commercial poultry production.

In general, populations of live sporulated oocysts from coccidial protozoa are obtained and treated by known mechanical, chemical or enzymatic procedures to disrupt the oocyst coat, thus allowing release of the sporocysts inside. The sporulated oocysts can

be used after treatment without further purification or the sporocysts can be isolated by methods known to those skilled in the art. The resulting sporocysts or treated sporulated oocysts are then placed in a pharmaceutically acceptable carrier, diluent or excipient. If desired, the preparation can contain a preservative such as a virocidic and/or a bacteriocide.

5 Additionally, the preparation can also contain immune stimulants, growth stimulants, and/or nutrients. The preparation and its carrier can be packaged alone or can be combined with one or more additional vaccines used in the class *Aves*. The preparation can be administered alone or in combination with anti coccidial drugs.

10 The preparation of the present invention is administered to newly hatched chicks by intra yolk sac injection. The method for administration of medicaments by intra yolk sac injection is described in U.S. Patent No. 5,311,841, herein incorporated by reference, and assigned to the common assignee of the present application. The injections can be accomplished manually or can be partially or fully automated by the use of suitable injection devices.

15 The preparation and method of the invention have the advantage of allowing the rapid administration of a controlled, effective dose of coccidial protozoa to prevent the outbreak of coccidiosis and/or lessen production losses due to coccidial infections in birds, especially domestic poultry. Production losses associated with infection by coccidial protozoa include, but are not limited to, decreases in weight gain and feed efficiency.

20 Traditionally, coccidiosis vaccines in poultry have been administered orally in the feed or water. The dose of vaccine administered, therefore, varies with the amount of feed or water consumed. The result is that some birds receive less than the optimum dose of vaccine while other birds are over dosed. In another method the vaccine is sprayed on the chicks. Alternatively, the vaccine can be administered by oral gavage. While this method

25 insures that each bird receives a dose of the vaccine, its disadvantages include high labor intensity, possible injury to the mouth of the bird, and improper administration of the vaccine resulting in aspiration of the vaccine into the respiratory tract. The present invention overcomes these problems. The use of intra yolk sac injection insures that each bird receives a measured, optimal dose of vaccine. The injection is minimally invasive,

30 with very low rates of injury to the birds. In addition, the rate of misinjection, that is the injection of the vaccine into sites other than the yolk sac, is also low.

An additional problem associated with oral vaccination is shedding. Orally vaccinated birds shed substantial numbers of coccidial oocysts in their feces. In the broiler industry where birds are housed on the floor, this can result in substantial parasite build-up in the floor litter. For this reason, the broiler industry has traditionally relied on chemotherapy rather than vaccination. The present invention has the advantage of resulting in substantially less shedding than oral vaccination. The present invention, therefore, is more applicable to broiler production than oral vaccination. The applicability of the present invention to the broiler industry will allow a decrease in the industry's reliance on drugs to control coccidiosis and so will lessen consumer concerns about drug residues in poultry.

In certain situations, such as when the environment contains a heavy parasite load, it would be desirable to provide protection by oral administration of anti coccidial drugs until immunity can be established. This is difficult to do with traditional oral vaccination, because the drugs kill the organisms in the vaccine preventing the development of resistance. An additional advantage of the present invention is that it is effective when given in combination with anti coccidial drugs.

In accordance with the invention described herein, a preparation and method are disclosed for the prevention and treatment of coccidiosis in birds. The invention has been shown to be as effective in preventing coccidiosis in birds as the traditional oral vaccination or the administration of anti coccidial agents in the feed or water. The invention is especially suited for application to commercial poultry production because it allows for the rapid administration of a controlled dose of the coccidiosis vaccine preparation to large numbers of birds. The invention uses a form of coccidial protozoa which is effective in conferring immunity at lower doses than the previously used oocyst stage when administered by intra yolk sac injection. The stage of protozoa used also has an advantage over the other commonly used stage of coccidial protozoa, sporozoites, in that the protozoa remains viable for extended periods under simple conditions.

Source of oocysts

Any source of oocysts of a single species can be used. Preferably, the oocysts are from a coccidial species of the order *Eucoccidiida*. More particularly, the coccidial oocysts of the genus *Eimeria*, *Tyzzeria* or *Wenyonella*. More particularly the coccidial

oocysts are of the species *E. acervulina*, *E. maxima*, *E. mitis*, *E. tenella*, *E. necatrix*, *E. brunetti*, *E. praecox*, *E. adenoeides*, *E. dispersa*, *E. gallopavonis*, or *E. meleagritidis*.

In one embodiment, 14 to 28 day-old leghorn chickens are inoculated by oral gavage with approximately $4-8 \times 10^3$ sporulated oocysts from a field isolate of the
5 coccidial species of interest. In one embodiment, specific pathogen free (SPF) chickens are used. Alternatively, a clonal strain of a coccidial species can be used. A clonal strain is one derived from a single oocyst. Excreta are collected from 4 days to 9 days after inoculation. Either attenuated or unattenuated, fully infective strains of coccidial protozoa can be used. In the preferred embodiment, unattenuated protozoa are used.

10 Isolation of oocysts

Various methods for the isolation of coccidial oocysts from excreta are known in the art and will only be briefly described herein. The initial step is separation of the oocysts from extraneous material. In one method, excreta is mixed with a minimum of 2
volumes (w/v) of saturated aqueous NaCl to form a slurry. If necessary, the slurry can be
15 processed in a mixer or blender until a homogenous consistency is achieved. The slurry is centrifuged at about $800 \times g$ for 10 minutes at 4°C . The supernate is collected by pouring through a double layer of 24 x 24 weave cheese cloth. The filtered supernate is diluted with two volumes of potable water and centrifuged at about $1600 \times g$ for 10 minutes at 4°C . The pelleted oocysts are washed with water and pelleted by centrifugation as
20 described an additional three times. The oocysts are then washed three times in 2.5% potassium dichromate using the same procedure used for the water washes. After the final wash, the oocysts can be stored in 2.5% potassium dichromate at 4°C or transferred to a container for sporulation.

In an alternative procedure, the excreta is placed in a container at about $40-50^\circ\text{F}$
25 and mixed with water at a ratio of about 2 pounds of excreta per gallon of water. The mixture is agitated for form a slurry and then pumped into a vibrating sieve fitted with screens of between 50 to 250 mesh to remove the large particulate matter. The material passing through the seive is collected and pumped into a continuous flow centrifuge also maintained at about $40-50^\circ\text{F}$. The resulting centrate is discarded while the solid material
30 containing the oocysts is collected and added to an equal volume of concentrated sucrose or high fructose corn syrup (HFCS). To this is added an equal volume of water for a total

final volume of about four times the volume of the initial solids. The specific gravity of the final mixture is preferably sufficient to float the oocysts, preferably between about 1.01 to 1.2 g/l and more preferably about 1.09 g/l. The final mixture is then pumped into a continuous centrifuge at a rate to allow the oocysts to remain in the centrate. The solids are discarded. Concentration of the oocysts and removal of the residual sugar can be accomplished by continuous flow centrifugation at a high feed rate which allows separation of the water phase which contains the oocysts from the sugar phase.

Alternatively, the material can be centrifuged in a rotor centrifuge. In this case, the supernatant is discarded and the oocysts in the resulting pellet are resuspended in water.

Additionally, the residual sugar can be removed by filtration using filters with a pore size which excludes the oocysts. When filtration is used, tangential flow is preferred. The isolated oocysts are placed in sterile water or 2.5% potassium dichromate and stored at 2-8°C until sporulation.

Sporulation

Various methods to induce sporulation are known to those skilled in the art. For example, sporulation can be accomplished by placing oocysts in an aqueous solution of 2.5% potassium dichromate in a container and placing a cotton or foam rubber plug in the opening. The container is agitated on an orbital shaker at 250 rpm and 28-30°C for 48-72 hours. Alternatively, the solution containing the oocysts can be constantly agitated while air is bubbled through the solution at about 25 to 75 cubic feet per hour. During the sporulation process, samples are withdrawn at regular intervals and tested for sporulation by microscopic examination of the oocysts to determine if they contain sporocysts.

Alternatively, sporulation can be accomplished by incubating oocysts in a solution containing an oxidizing agent with a redox potential of greater than 0.5 V at about 20-38°C with constant agitation. Examples of suitable oxidizing agents include potassium permanganate, potassium perchlorate, sodium hypochlorite, and hydrochlorous acid which have redox potentials of approximately 1.49 V, 1.37 V, 1.49 V, and 1.63 V, respectively. The amount of oxidizing agent added will vary with the agent used and the species of protozoa and can be determined empirically. For protozoa of the genus *Eimeria*, preferred concentrations of oxidizing agents include 0.1 to 0.75 wt% for potassium perchlorate, 0.5 to 2.9 wt% for potassium permanganate, 0.001 to 0.1 wt% sodium hypochlorite and 1 ppm

to 5 ppm for hydrochlorous acid. During sporulation, oxygen is bubbled through the mixture at a rate sufficient to give an initial dissolved oxygen content of at least 50%.

Following sporulation, sporulated oocysts are collected by filtration, centrifugation or other acceptable concentration procedures known to those skilled in the art. For example, sporulated oocysts can be concentrated by centrifugation at about 1500 x g for 10 minutes at 4°C and the supernate discarded. After concentration, sporulated oocysts may be sterilized by use of a chemical disinfectant. The chemical used for sterilization should be one which will kill bacteria and viruses, but will not kill the sporulated oocysts. In particular, the disinfectant used should kill the infectious bursal disease (IBD) and chick anemia (CAV) viruses under the conditions used, for example by suspension in 2-5% sodium hypochlorite for 2-30 minutes. Following disinfection, sodium hypochlorite is removed by four to six washes with water followed by centrifugation at 1500 x g for 10 minutes preferably at 4°C. Alternatively, washing can be accomplished by filtration, preferably tangential flow filtrations, using several volumes of sterile water. After washing, the sporulated oocysts can be stored in a suitable medium including but not limited to, water alone, 0.5X PBS, or a solution containing a suitable disinfectant or preservative such as 2.5% potassium dichromate, 0.5X phosphate buffered saline (PBS) containing 30 µg/ml gentamicin, 0.1 wt% potassium perchlorate, 0.001 wt% sodium hypochlorite, 1 ppm hydrochlorous acid, or 1 mM sodium hydroxide.

20 Treatment of sporulated oocysts

Methods for treatment of sporulated oocysts to disrupt the oocyst wall are well known to those skilled in the art and will only be briefly described herein. In one embodiment, the oocyst wall is removed by mechanical disruption. Any method which exerts sufficient mechanical force on the oocyst wall to cause its rupture without destroying the sporocyst within can be used. Typically, this is achieved by mixing sporulated oocysts with glass beads. In one embodiment, sporulated oocysts are diluted to a concentration of about 2×10^6 /ml in PBS. An amount of the 2×10^6 /ml suspension of sporulated oocysts is added to a container containing 0.5 mm glass beads such that the ratio of sporulated oocysts to glass beads is approximately 12:1. The container containing the sporulated oocysts and the glass beads is agitated at high speed, for example by vortexing, for five minutes and then chilled on ice.

Following vortexing, the supernate is removed and saved. The glass beads are then washed with PBS and the supernate saved and combined with the supernate obtained after vortexing. The supernate is then centrifuged at about 9020 x g for about two minutes and the supernate discarded. The pellet is resuspended in 0.2 ml of PBS. A sample of the resuspended pellet can be viewed using a microscope to determine the extent of oocyst wall disruption as measured by the number of sporocysts present. Alternatively, the dosage can be calculated based on the number of sporulated oocysts treated. Although the pellet preferably should contain primarily sporocysts, it will be apparent to those skilled in the art that intact oocysts, empty oocyst coats, and sporozoites may also be present.

Although not necessary to practice the invention, those skilled in the art will realize that it is possible to further purify the preparation to increase the proportion of sporocysts present using methods commonly known in the art. For example and without limitation, sporocyst isolation can be accomplished by centrifugation using 50% Percoll as described by Dulski and Turner, *Avian Diseases* 32(2):235-239 (1988), herein incorporated by reference. The concentration of protozoa per ml is then determined by using a hemacytometer or other method of determining cell number.

Alternatively, the oocyst wall can be disrupted by either chemical or enzymatic treatment. As with mechanical disruption, any chemical or enzymatic treatment that results in the rupture of the oocyst wall, but maintains the viability of the sporocyst contained within the sporulated oocyst can be used.

Following treatment, the coccidial parasites are sanitized by any suitable method. Any method that results in the destruction of microbial contaminants, but does not significantly decrease the viability of the coccidial parasites can be used. Generally, sanitation is accomplished by the use of a chemical disinfectant. In one embodiment, sanitation is by suspension in sodium hypochlorite followed by washing with sterile water as described above.

Sporulated oocysts which have been treated to disrupt the oocyst wall as described above are then diluted with a pharmaceutically acceptable carrier, diluent or excipient to the desired concentration. Preparations can be formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The preparation may be an injectable solution or suspension in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and

solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. The vehicles and solvents used may optionally include a buffering agent, such as phosphate buffer. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed, including
5 synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid are useful in the preparation of injectables. Dimethyl acetamide, surfactants including ionic and non-ionic detergents, and polyethylene glycols can be used. Mixtures of solvents and wetting agents such as those discussed above are also useful. Other possible formulations will be apparent to those skilled in the art. Formulation of drugs is discussed in, for example,
10 Hoover, John E., *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, Pennsylvania (1975), and Liberman, H.A. and Lachman, L., Eds., *Pharmaceutical Dosage Forms*, Marcel Decker, New York, N.Y. (1980).

If the preparation is to contain multiple species of coccidial protozoa, appropriate numbers of treated oocysts from each species are combined to form a single preparation.
15 The preparation can be used immediately to vaccinate birds or it can be packaged into suitable single-dose or multiple-dose containers. If desired, the preparation can be lyophilized.

Testing for Microbial Contamination

Because the preparation is to be injected into the yolk sac, it is preferred that the
20 preparation be substantially free of extraneous viable bacteria, fungi and viruses affecting birds. In particular, it is preferred that the preparation be substantially free of *Salmonella*, infectious bursal disease virus (IBDV), and chick anemia virus (CAV). To insure that the preparation is substantially free of *Salmonella*, infectious bursal disease virus and chick anemia virus as defined herein, it is not necessary to test every lot of the preparation
25 produced. However in one preferred embodiment, each lot is tested for *Salmonella*. In another preferred embodiment, testing of every fifth lot is sufficient for IBDV and CAV. As used herein the term lot refers to a collection of closed, final containers or other final dosage units that are expected to be homogeneous and equivalent with respect to contamination during filling or preparation of the final product. The dosage units of a lot
30 are filled or otherwise prepared from the same final bulk vaccine closed in one final lot.

Methods for testing for extraneous bacterial, viral or fungal contamination are known to those skilled in the art. One preferred method of testing is set forth in 9 C.F.R. 113.27(1999), hereby incorporated by reference.

To test for bacterial contamination, a 0.2 ml sample from each of 10 final
5 containers of the preparation of the present invention are inoculated into corresponding individual vessels containing at least 120 ml of soybean casein digest medium. The containers are then incubated at 30 to 35°C for 14 days. To test for fungal contamination, a 0.2 ml sample from each of 10 final containers of the preparation of the present invention are inoculated into corresponding individual vessels containing at least 40 ml of soybean
10 casein digest medium. The vessels are incubated at 20 to 25°C for 14 days. After the incubation period, the vessels are examined macroscopically for microbial growth. If growth cannot be determined reliably by visual examination, the judgment is confirmed by microscopic examination. If growth is found in at least 2 vessels, a retest is conducted using samples from 20 final containers to rule out faulty technique. If no growth is found
15 in at least 9 vessels in the initial test or at least 19 vessels in a retest, the preparation is considered substantially free of extraneous bacterial and fungal contamination.

It is also preferred that the preparation be substantially free of Salmonella. Salmonella is a bacterium commonly found in the gastrointestinal tract of poultry. Thus, Salmonella contamination is a concern when, as in the present invention, some of the
20 material used in producing the preparation is obtained from the feces of birds.

Methods for detecting Salmonella contamination are well known to those skilled in the art. One preferred method for detection of Salmonella contamination is contained in 9 C.F.R. 113.30 (1999), hereby incorporated by reference. Briefly, samples of the preparation are obtained before the addition of bacteriostatic or bactericidal agents. Five
25 ml of the preparation is then inoculated into 100 ml of liquid broth medium (tryptose and either selenite F or tetrathionate). The inoculated medium is incubated at 35 to 37°C for 18-24 hours. After incubation, samples of inoculated medium are transferred to either MacConkey agar or Salmonella-Shigella agar and incubated for 18-24 hours. If no growth typical of Salmonella is noted, the plates are incubated for an additional 18 to 24 hours and
30 again examined. If no growth of Salmonella is noted at the second examination, the preparation is considered substantially free of Salmonella contamination.

In another preferred embodiment, the preparation is substantially free of Chicken Anemia Virus. Chicken Anemia Virus causes anemia in young chicks and was first isolated from contaminated vaccines in Japan (Yuasa, et al., *Avian Diseases*, 23:366-385 (1979)). Chicken Anemia Virus has been found to be resistant to heating to 70°C for 1
5 hour, heating to 80°C for 5 minutes, exposure to pH 3.0 and exposure to either chloroform or ether (Yuasa, et al., *Avian Diseases*, 23:366-385 (1979)). Contamination of vaccine preparations with the Chicken Anemia Virus is a concern due to the hardness of the virus. Prior art vaccine preparations have failed to address the possibility of CAV contamination.

The method of testing for Chicken Anemia Virus is based on that contained in 9
10 C.F.R. 113.47 (1999) herein incorporated by reference. MSB-1 cells from the Maine Biological Laboratories, Waterville, ME are used as the indicator cell line for Chicken Anemia Virus. MSB-1 cells are a lymphoblastoid cell line from a Marek's disease lymphoma that show cytopathic effect when infected with Chicken Anemia Virus. Cells are maintained in Opti-MEM® (Life Technologies, Gaithersburg, MD) or other suitable
15 media at 41°C for at least 24 days prior to testing. Cells are subcultured 10-12 times during the maintenance period with all but the last subculture resulting in a monolayer of at least 75 cm². The last subculture is at least 6 cm².

Three groups of MSB-1 monolayers are used for each test, a negative control group, a positive control group, and a test group. At the start of the 24 day maintenance
20 period, the positive control group is inoculated with 10^{5.75} TCID₅₀/ml of Chicken Anemia Virus, Del Ros strain originally obtained from the Center for Veterinary Biologics Laboratory (Ames, IA) and the test group inoculated with 1 ml of the test preparation. The negative control group is inoculated with 1.0 ml of a preparation known to be free of Chicken Anemia Virus. The cells are then maintained for at least 24 days as described
25 above.

Two days after the last subculture, the three groups of monolayers are fixed and treated with a specific chicken polyclonal Chicken Anemia Virus antibody (Hy-Vac, Adel, IA). The monolayers are then washed and treated with a fluorescein labeled goat anti chicken IgG (H&L, Jackson ImmunoResearch, West Grove, PA) and examined for specific
30 fluorescence. If the positive control shows specific fluorescence and there is no difference in fluorescence between the test and negative control groups, the preparation is considered substantially free of Chicken Anemia Virus.

Alternatively, the presence of Chicken Anemia Virus can be detected by the polymerase chain reaction (PCR). Three days after the last subculture, DNA is extracted from the three groups of monolayers using well established procedures. See, for example, Ausubel et al., *Short Protocols in Molecular Biology*, 2nd Ed., John Wiley & Sons, 1992; 5 Sambrook et al., *Molecular Cloning, A Laboratory Manual*, 2nd Ed. Cold Spring Harbor Laboratory Press, 1989; Davis, et al., *Basic Methods in Molecular Biology*, Elsevier, 1986. Briefly, cells are lysed by two cycles of freezing to -80°C and thawing at 37°C. Cellular debris is removed by centrifugation at about 3,500 x g for 20 minutes. The supernatant is treated with DNase and RNase A to remove cellular contaminants and the proteins and/or 10 virus precipitated with polyethylene glycol. The precipitate is treated with Proteinase K and extracted three times with phenol/chloroform/isoamyl alcohol. DNA is precipitated with sodium acetate-ethanol and pelleted by centrifugation at 14,000 x g for 15 minutes. The resulting pellet is resuspended in double distilled water and stored at -20°C.

Conserved regions of the Chicken Anemia Virus viral genome are amplified using 15 standard techniques (Innis et al., *PCR Protocols*, Academic Press, 1990). Information on the Chicken Anemia Virus viral genome for designing suitable primers can be found on databases well known to those in the biomedical arts such as the databases available through the U.S. National Institutes of Health website at <http://www.ncbi.nlm.nih.gov>, all herein incorporated by reference. PCR products are analyzed by agarose gel 20 electrophoresis and ethidium bromide staining. If the PCR amplification does not result in a band corresponding to the band found in the positive control, the preparation is considered substantially free of Chicken Anemia Virus.

Also preferably, the preparation is substantially free of Infectious Bursal Disease Virus (IBDV). IBDV affects the bursa of Fabricius of young chickens resulting in 25 decreased feed efficiency and weight gain, immunosuppression and increased mortality. As with CAV, IBDV contamination is a concern in vaccine preparations because IBDV is highly contagious and resistant to disinfectants. As with CAV, prior coccidiosis vaccine preparations have failed to insure that the vaccine is free of IBDV contamination.

The method for detection of IBDV in the preparation of the present invention is the 30 same as for Chicken Anemia Virus with the following changes. The cell line used for IBDV testing is a primary chick embryo fibroblast cell line. After addition of 1.0 ml of the preparation or $10^{8.25}$ TCID₅₀ of IBDV originally obtained from American Type Culture

Collection (ATCC VR-2041 strain D78), the monolayers are maintained in basal medium Eagle (BME) at 37°C and 5% CO₂ for at least 14 days. Detection is accomplished using an IBDV specific polyclonal chicken antiserum and a fluorescein labeled goat anti-chicken IgG (Jackson ImmunoResearch, West Grove PA). If the positive control shows specific
5 fluorescence and there is no difference in fluorescence between the test and negative control groups, the preparation is considered substantially free of IBDV.

Alternatively, the test for the IBDV contamination can be accomplished using PCR as described for Chicken Anemia Virus. Information on the IBDV viral genome for designing suitable primers can be found on databases well known to those in the
10 biomedical arts such as the databases available through the U.S. National Institutes of Health website at <http://www.ncbi.nlm.nih.gov>, all herein incorporated by reference. If the PCR amplification does not result in a band corresponding to the band found in the positive control, the preparation is considered substantially free of Infectious Bursal Disease Virus.

15 Administration

The preparation is administered by intra yolk sac injection. The method for administration of materials by intra yolk sac injection using either a needle and syringe or an automatic injection device is described in U.S. Patent No. 5,311,841, herein
incorporated by reference and assigned to the common assignee of the present invention.

20 Briefly, the preparation to be administered is introduced into the yolk sac through a hypodermic needle inserted in an area having a diameter of about 1 cm on the ventral surface of the chick with the navel being located approximately half-way between the center of the target area and its 12 o'clock position. In the preferred embodiment, administration of the preparation is accomplished within about 48 hours of hatching.

25 The number of sporocysts injected will vary with known factors such as species of protozoa, age of bird and husbandry conditions. For example, birds housed under conditions, such as floor litter, will "self vaccinate" by consumption of shed oocyst and so will require a smaller dose than birds housed under conditions in which ingestion of shed oocysts is limited. Exact numbers of sporocysts required can easily be determined by
30 those of ordinary skill in the art without undue experimentation.

In one embodiment, at least about 50, sporocysts of a species of coccidial protozoa are administered to newly hatched chicks. In another embodiment, at least about 50 sporocysts of multiple species of coccidial protozoa are administered in a single dose. In yet another embodiment, the preparation of the invention contains at least about 50
5 sporocysts of each of the coccidial species *E. acervulina*, *E. maxima*, and *E. tenella* for a total of at least 150 sporocysts in a single dose. In another embodiment, at least about 1000, sporocysts of a species of coccidial protozoa are administered to newly hatched chicks. In still another embodiment, at least about 1000 sporocysts of multiple species of coccidial protozoa are administered in a single dose. In yet another embodiment, the
10 preparation of the invention contains at least about 1000 sporocysts of each of the coccidial species *E. acervulina*, *E. maxima*, and *E. tenella* for a total of at least 3000 sporocysts in a single dose. In another embodiment, the preparation contains at least about 5,000 sporocysts of *E. tenella*, at least about 5,000 sporocysts of *E. maxima*, and at least about 10,000 sporocysts of *E. acervulina*, in a single dose. In yet another embodiment, the
15 preparation contains at least about 10,000 sporocysts of *E. tenella*, at least about 10,000 sporocysts of *E. maxima*, and at least about 40,000 sporocysts of *E. acervulina* in a single dose. In another preferred embodiment, the preparation contains at least about 20,000 sporocysts of *E. tenella*, at least about 20,000 sporocysts of *E. maxima*, and at least about 50,000 sporocysts of *E. acervulina*, in a single dose.

20 As described previously, sporulated oocysts are treated to disrupt the oocyst wall. Therefore in one embodiment, at least about 50 treated oocysts of a species of coccidial protozoa are administered to newly hatched chicks. In another embodiment, at least about 50 treated oocysts of multiple species of coccidial protozoa are administered in a single dose. In yet another embodiment, the preparation of the invention contains at least about
25 50 treated oocysts of each of the coccidial species *E. acervulina*, *E. maxima*, and *E. tenella* for a total of at least 150 treated oocysts in a single dose. In another embodiment, at least about 1000 treated oocysts of a species of coccidial protozoa are administered to newly hatched chicks. In still another embodiment, at least about 1000 treated oocysts of multiple species of coccidial protozoa are administered in a single dose. In yet another
30 embodiment, the preparation of the invention contains at least about 1000 treated oocysts of each of the coccidial species *E. acervulina*, *E. maxima*, and *E. tenella* for a total of at least 3000 treated oocysts in a single dose. In still another embodiment, the preparation

contains at least about 5,000 treated oocysts of *E. tenella*, at least about 5,000 treated oocysts of *E. maxima*, and at least about 10,000 treated oocysts of *E. acervulina* in a single dose. In yet another embodiment, the preparation contains at least about 10,000 treated oocysts of *E. tenella*, at least about 10,000 treated oocysts of *E. maxima*, and at least about 40,000 treated oocysts of *E. acervulina*, in a single dose. In another embodiment, the preparation contains at least about 20,000 treated oocysts of *E. tenella*, at least about 20,000 treated oocysts of *E. maxima*, and at least about 50,000 treated oocysts of *E. acervulina* in a single dose.

As discussed previously, although it is possible to isolate the sporocysts released following treatment of the sporulated oocysts to disrupt the oocyst wall, it is not necessary to do so to practice the present invention. Therefore, the preparation may contain intact oocysts, empty oocyst coats, and sporozoites in addition to sporocysts. In determining the percentage of sporocysts present in the preparation, a sample is obtained and the number of coccidial organisms present determined. The total number of coccidial protozoa present can be counted by any method known in the art, for example by microscopic examination. Intact oocysts, intact sporocysts and free sporozoites are each counted as one protozoan. Empty coats are not counted. Thus, in one preferred embodiment, at least 10% of the total coccidial protozoa in the preparation are sporocysts. In another embodiment, at least 25% of the coccidial protozoa in the preparation are sporocysts. In still another preferred embodiment, at least 50% of the coccidial protozoa in the preparation are sporocysts. In yet another embodiment, at least 70% of the coccidial protozoa in the preparation are sporocysts. In still another preferred embodiment, at least 95% of the coccidial protozoa in the preparation are sporocysts.

The volume administered can vary, but preferably contains a sufficient number of coccidial organisms to result in an immune response. As will be recognized by those in the art, the exact volume administered will vary with well known factors, such as, the concentration of coccidial organisms in the preparation, the accuracy of the injection equipment used, and the size of the animal. In general, the volume administered should be large enough so that it can be accurately measured, but not so large as to cause injury to the animal. For intra yolk sac administration, injection volumes are preferably between 0.01 ml and 5 ml, more preferably between 0.025 ml and 1 ml and more preferably still between 0.05 ml and 0.5 ml.

In further embodiment, the preparation contains a preservative such as a bacteriocide, virocid e or combination of the two. Suitable preservatives, include, but are not limited to Amphotericin B, Nystatin, tetracycline, penicillin, streptomycin, polymyxin B, neomycin, gentamicin, potassium perchlorate, sodium hypochlorite, hydrochlorous acid, and sodium hydroxide. Approximate concentrations of preservative are Amphotericin B (30 units/ml), Nystatin (30 µg/ml), tetracycline (30 µg/ml), penicillin (30 units/ml), streptomycin (30 µg/ml), polymyxin B (30 µg/ml), neomycin (30 µg/ml), gentamicin (30 µg/ml), 0.1 wt% potassium perchlorate, 0.001 wt% sodium hypochlorite, 1 ppm hydrochlorous acid, or 1 mM sodium hydroxide. In a preferred embodiment, the preservative is gentamicin. In a more preferred embodiment, the preservative is gentamicin at a concentration of about 30 µg/ml.

In another embodiment, the preparation contains or is administered in conjunction with at least one immune system stimulant. When administered in conjunction with the preparation, the immune stimulant can be administered at the same time, at a time prior to, or at a time after administration of the preparation. When administered at the same time as the preparation, the immune system stimulant can be contained in the preparation or be administered separate from the preparation. Administration of the immune stimulant can be by any suitable means known in the art, including, but not limited to oral and parenteral routes. Suitable immune system stimulants include, but are not limited to, cytokines, growth factors, chemokines, mitogens and adjuvants. Suitable immune stimulants are well known to those skilled in the art and can be found, for example, in Plotkin and Orenstein, *Vaccines*, Third Ed., W.B. Saunders, 1999; Roitt et al., *Immunology*, Fifth Ed., Mosby, 1998; and Brostoff, et al., *Clinical Immunology*, Gower Medical Publishing, 1991. Examples of immune stimulants, include, but are not limited to, Alum (aluminum phosphate or aluminum hydroxide), Freund's adjuvant, calcium phosphate, beryllium hydroxide, saponins, polyanions, e.g. poly A:U, Quil A, inulin, lipopolysaccharide endotoxins, liposomes, lysolecithins, zymosan, propionibacteria, mycobacteria, and cytokines, such as, interleukin-1, interleukin-2, interleukin-4, interleukin-6, interleukin-12, interferon-α, interferon-γ, granulocyte-colony stimulating factor. In one embodiment, the immune stimulant is *Propionibacteria acnes*.

In another embodiment, the preparation contains or is administered in conjunction with a vaccine preparation for at least one additional disease of birds. As used herein,

vaccine preparations include, but are not limited to, live, inactivated, attenuated, subunit vaccine preparations or any combination thereof. Administration can be by any suitable means known in the art. A list of suitable vaccine preparations includes, but is not limited to vaccine preparations for fowl cholera, colibacillosis, fowl pox, infectious bronchitis, fowl typhoid, viral hepatitis, encephalomyelitis, infectious bursal disease, laryngotracheitis, duck plague, hemorrhagic enteritis, leukosis complex, Marek's disease, lymphoid leukosis, reticuloendotheliosis, lymphoproliferative disease, Newcastle disease, and viral arthritis.

The preparation can also contain or be administered in conjunction with at least one growth stimulant. When administered in conjunction with the preparation, the growth stimulant can be administered at the same time, at a time prior to, or at a time after administration of the preparation. When administered at the same time as the preparation, the growth stimulant can be contained in the preparation or be administered separately. Administration of the growth stimulant can be by any suitable means known in the art, including, but not limited to oral and parenteral routes. Possible growth stimulants include, without limitation, growth hormone, growth hormone releasing hormone, insulin-like growth factor I, insulin-like growth factor II, avian interleukins, nerve growth factor, thyroxine releasing hormone, thyroxine stimulating hormone, monoiodotyrosine, diiodotyrosine, triiodotyrosine, thyroxine and corticosterone.

In another embodiment, the preparation can contain or be administered in conjunction with at least one nutrient, for example and without limitation, vitamins, minerals, amino acids, sugars and fatty acids. When administered in conjunction with the preparation, the nutrient can be administered at the same time, at a time prior to, or at a time after administration of the preparation. When administered at the same time as the preparation, the nutrient can be contained in the preparation or be administered separately. Administration of the nutrient can be by any suitable means known in the art, including, but not limited to oral and parenteral routes.

In yet another embodiment, the preparation can be administered in conjunction with an anti coccidial drug administered orally in the feed and/or water. The list of anti coccidial drugs which may be suitable for administration in conjunction with the preparation includes, but is not limited to, sulfaquinoxaline, nitrofurazone, sodium arsanilate, arsanilic acid, butynorate, nicarbazin, furazolidone, nitromide, sulfantran,

roxarasone, oxytetracycline, amprolium, chlortetracycline, ethopabate, buquinolate, clopidol, meticlorpindol, decoquinate, sulfadimethoxine, ormetoprin, monensin, robenidine, lasalocid, salinomycin, methyl benzoate halofuginone, narasin, madurimicin, semduramicin, and zoalene. In one preferred embodiment anti coccidial drugs approved by the U.S. Food and Drug Administration for administration to poultry are used. Examples of approved drugs include monensin (Coban), salinomycin (Bio-Cox, Sacox), maduramicin (Cygro), semduramicin (Aviax), lasalocid (Avatec), narasin (Monteban), amprol (Amprolium), nicarbazin (Nicarb), zoalene (Zoamix), clopidol (Coyden), robenidine (Robenz), halofuginone (Stenerol), narasin in combination with nicarbazin (Maxiban), and methyl benzoate (Decox). In a more preferred embodiment the anti coccidial drugs administered are ionophores, for example, monesin (Coban), salinomycin (Bio-Cox, Sacox), maduramicin (Cygro), semduramicin (Aviax), lasalocid (Avatec), narasin (Monteban). Anti coccidial drugs are administered in the feed at the manufacturer's recommended dosage, for example, monesin (Coban) (90-110 g/ton), lasalocid (68-113 g/ton or 0.0075-0.0125%), salinomycin (40-60 g/ton), maduramicin (4.54-5.45 g/ton or 5-6 ppm), semduramicin (22.7 g/ton or 25 ppm) and narasin (54-72 g/ton).

EXAMPLES

The following examples are intended to provide illustrations of the application of the present invention. The following examples are not intended to completely define or otherwise limit the scope of the invention.

Example 1

Effect of Intra Yolk Sac Administration of Treated

E. tenella Oocysts on Post Challenge Lesion Scores at Day 21

Treated oocysts of the species *E. tenella* were prepared as previously described. The treated (ground) oocysts were sanitized with 5.25% hypochlorite, washed five times in water, placed in sterile water and administered by intra yolk sac injection on day 0. The treatments shown in Table 1 were used in a randomized block experimental design. There were 12 pen replicates of 10 specific pathogen free (SPF) birds per pen housed in two battery rooms. The birds were fed a diet formulated to meet or exceed the National

Research Council recommended nutrient specifications. Birds were challenged by oral gavage of approximately 40,000 sporulated oocysts on day 15 of the study. Birds were sacrificed on day 21 and lesion scores determined. Data were analyzed by analysis of variance (ANOVA procedure of SAS). Mean separation was by least square comparison.

- 5 The results are shown in Fig. 1. In all cases, lesion scores for IYS injected birds were lower than for challenged birds receiving either sham IYS injection or oral vaccination. Lesion scores were significantly lower ($P<0.05$) in birds receiving 20,000 or 50,000 treated oocysts by IYS injection.

Table 1

10	Treatment Number	Treatment	Challenge
	1	IYS sham	No
	2	IYS sham	Yes
	3	Oral 1,000 intact oocysts	Yes
	4	IYS 1,000 treated oocysts	Yes
15	5	IYS 5,000 treated oocysts	Yes
	6	IYS 10,000 treated oocysts	Yes
	7	IYS 20,000 treated oocysts	Yes
	8	IYS 50,000 treated oocysts	Yes

Example 2

- 20 Effect of Intra Yolk Sac Administration of Intact or Treated *E. tenella* Oocysts on Post Challenge Feed Efficiency

- The experimental design was as described in Example 1 except the study used 48 floor pens of 50 birds per pen. The treatments consisted of sham injection, with or without challenge, and administration of increasing numbers of either intact or treated oocysts of *E. tenella* as shown in Table 2. Feed to gain ratios, a measure of feed efficiency, were determined between days 14 to 21. As shown in Fig. 2, in all cases the feed to gain ratio was lower in birds injected with treated oocysts than with intact oocysts. Intra yolk sac administration of 20,000 treated oocysts resulted in feed to gain ratios that were not

different ($P>0.05$) from those in unchallenged control birds. Fifty thousand intact oocysts were required to achieve the same result as with 20,000 treated oocysts.

Table 2

	Treatment Number	Treatment	Challenge
5	1	IYS sham	No
	2	IYS sham	Yes
	3	IYS 5,000 intact oocysts	Yes
	4	IYS 5,000 treated oocysts	Yes
	5	IYS 20,000 intact oocysts	Yes
10	6	IYS 20,000 treated oocysts	Yes
	7	IYS 50,000 intact oocysts	Yes
	8	IYS 50,000 treated oocysts	Yes

Example 3

Effect of Intra Yolk Sac Administration of Intact or Treated

15 *E. tenella* Oocysts on Post Challenge Gain Between Days 14-21

Experimental design and treatment groups were as described in Example 2. Total gain was measured between days 14 to 21 of the study. The results are shown in Fig 3. In all cases, birds receiving treated oocysts had higher post challenge gains than birds receiving the same number of intact oocysts. In birds receiving either 20,000 or 50,000 treated oocysts, the gain was not significantly different ($P>0.05$) from unchallenged control birds.

Example 4

Effect of Intra Yolk Sac Administration of Treated

E. maxima Oocysts on Post Challenge Lesion Scores at Day 29

25 The preparation of oocysts was as previously described in Example 1. The study was conducted in battery cages with 12 replicate cages of 10 birds per cage. Birds were vaccinated on day 0, challenged on day 23 and sacrificed on day 29. The treatment groups

are described in Table 3. The results of the study are shown in Fig. 4. Birds given either 10,000 or 30,000 treated oocysts tended to have slightly higher lesion scores than did birds given the same number of intact oocysts. Birds receiving 20,000 treated oocysts, however, had lesion scores which were not significantly different ($P>0.05$) from unchallenged

5 controls.

Table 3

Treatment Number	Treatment	Challenge
1	IYS sham	No
2	IYS sham	Yes
3	IYS 10,000 intact oocysts	Yes
4	IYS 10,000 treated oocysts	Yes
5	IYS 20,000 intact oocysts	Yes
6	IYS 20,000 treated oocysts	Yes
7	IYS 30,000 intact oocysts	Yes
8	IYS 30,000 treated oocysts	Yes

Example 5

Effect of Intra Yolk Sac Administration of Intact or Treated *E. maxima* Oocysts on Post Challenge Feed Efficiency

The experimental design and treatments were the same as in Example 4. Feed to gain ratios were determined between days 23 and 29 of the study. The results of the experiment are shown in Fig 5. Birds given treated oocysts tended to have lower feed to gain ratios (greater feed efficiency) than did birds given intact oocysts. This difference was statistically significant ($P<0.05$) at the 20,000 and 30,000 dose levels. Birds receiving 20,000 treated oocysts had a feed to gain ratio that was not different from unchallenged controls.

Example 6

Effect of Intra Yolk Sac Administration of Intact or Treated

E. maxima Oocysts on Post Challenge Gain Between Days 23-29

The experimental design and treatment groups were as described in Example 4.

- 5 Total gain was measured between days 23 and 29. As shown in Fig. 6, gain tended to be higher in birds injected with treated oocysts as compared to birds injected with intact oocysts. This difference was statistically significant ($P < 0.05$) at the 20,000 and 30,000 oocyst dose levels.

Example 7.

- 10 Effect of Oral or Intra Yolk Sac Vaccination on the
Rate of Subsequent Oocyst Shedding by Vaccinated Birds

- Birds were administered 250,000 intact *E. tenella* oocysts either by oral gavage or intra yolk sac injection. Fecal oocysts were collected and counted 4, 7, 9 and 12 days after treatment. The results are summarized in Fig 7. Birds receiving oocysts by intra yolk sac
15 injection shed significantly fewer oocysts in their feces on days 4, 7 and 9 than did orally gavaged birds. It is important to note in Fig. 7 that the scale for shedding following oral gavage is x 10,000 while for intra yolk sac injection it is x 1,000.

Example 8

- 20 Effect on Lesion Score of Vaccination by Intra
Yolk Sac Injection of Treated Oocysts of
E. tenella on Birds Housed on Used Litter

- This experiment was designed to test the effectiveness of vaccination against coccidiosis by intra yolk sac injections under conditions similar to those seen in commercial poultry production. Oocysts for intra yolk sac injection were prepared as
25 described in Example 1. Birds were housed in 64 pens of 40 birds each. Pen litter was from a previous study and contained low levels of residual oocysts (approx. 1000 oocysts/g litter). This approximated commercial conditions in which litter is commonly used with several flocks of birds. This level of oocyst contamination was insufficient to confer resistance (see treatment group 3, sham vaccinated). Birds were challenged on day
30 28 of the study by feeding sporulated oocysts on ground corn to give an approximate

challenge of dose 75,000 oocysts per bird depending on feed intake. Treatment groups are described in Table 4. Treatments consisted of unvaccinated controls, birds receiving increasing doses of treated oocysts by intra yolk sac injection, birds vaccinated by oral administration of *E. tenella*, and birds treated with the coccidiostat Bio-Cox®

5 (Salinomycin, 60 ppm). The results are shown Fig 8. At all doses tested, there was no significant difference ($P>0.05$) in lesion scores between birds vaccinated by intra yolk sac injection, unchallenged controls, birds vaccinated by oral vaccination and birds receiving a coccidiostat. Thus, intra yolk sac injection of treated oocysts was as effective in providing protection against coccidiosis as were traditional methods of protection.

10 Table 4

Treatment Number	Treatment	Challenge
1	No vaccination	No
2	No vaccination	Yes
3	IYS sham	Yes
4	IYS 5,000 treated oocysts	Yes
5	IYS 10,000 treated oocysts	Yes
6	IYS 20,000 treated oocysts	Yes
7	Oral 1,000 oocysts	Yes
8	Coccidiostat (Bio-Cox®)	Yes

20

Example 9

Effect on Weight Gain of Vaccination by Intra Yolk Sac Injection of Treated Oocysts of *E. tenella* on Birds Housed on Used Litter

The experimental design and treatments were the same as described in Example 8.
25 Weight gain was determined during the week following challenge. The results are shown in Fig. 9. Weight gains for birds receiving 5,000 treated oocysts by intra yolk sac injection were significantly ($P<0.05$) higher than for unvaccinated, challenged controls (trt. 2). There was no significant difference in weight gain among birds receiving 10,000 or 20,000

treated oocysts by intra yolk sac injection, and birds vaccinated by the oral gavage method or receiving a coccidiostat. These results also show that intra yolk sac vaccination with treated oocysts is as effective as traditional methods under commercial conditions.

Example 10

5 Effect on Feed to Gain Ratio of Vaccination by
 Intra Yolk Sac Injection of Treated Oocysts of
 E. tenella on Birds Housed on Used Litter

 The experimental design and treatment groups were the same as described in
Example 8. The results are shown in Fig. 10. There was no significant difference
10 (P>0.05) in feed conversion as measured by feed to gain ratio between birds vaccinated
 with all three doses of treated oocysts by intra yolk sac injection and birds vaccinated by
 oral gavage or birds receiving a coccidiostat. Birds that were challenged and either
 received no vaccination or sham intra yolk sac vaccination had significantly poorer feed
 conversion as evidenced by higher feed to gain ratios. Again, these results show that intra
15 yolk sac injection of treated oocysts is as effective as traditional methods in providing
 protection against coccidiosis under commercial conditions.

Example 11

 Effect of Salinomycin on the Development of Resistance as
 Determined by Weight Gain to *E. tenella* Following
20 Oral or Intra Yolk Sac Immunization

 This experiment was designed to determine the effectiveness of IYS vaccination
 administered in combination with an anti coccidial drug. The experimental design was
 similar to Example 1 with the following differences. The experiment used Peterson
 Hubbard male broilers. Birds were housed in battery cages. All but one treatment group
25 were challenged by oral administration of approximately 40,000 sporulated oocysts on day
 23 of the study. Weight gain between days 21 and 28 was determined. Treatment groups
 are described in Table 5. Treatments consisted of unvaccinated controls, vaccination by
 intra yolk sac injection with or without concurrent oral administration of an anti coccidial
 drug, and oral vaccination with or without concurrent administration of an anti coccidial
30 drug. The anticoccidial drug was Salinomycin (SACOX®) administered in the feed

according to the manufacturer's instructions (60 ppm). The results are shown in Fig. 11. There was no difference in weight gain between unvaccinated, unchallenged controls and birds receiving anti coccidial drugs alone, oral vaccination without the anti coccidial drug, and intra yolk administration of treated sporulated oocysts with or without drugs.

- 5 Administration of the anti coccidial drug in combination with oral vaccination resulted in a significant ($P \leq 0.05$) decrease in weight gain. These results indicate that concurrent administration of anti coccidial drugs does not decrease the effectiveness of intra yolk sac vaccination but does decrease the efficacy of oral vaccination.

Table 5

10	Treatment Number	Treatment	Challenge
	1	No vaccination	No
	2	No vaccination	Yes
	3	No vaccination plus Salinomycin	Yes
	4	Oral 1,000 oocysts	Yes
15	5	Oral 1,000 oocysts plus Salinomycin	Yes
	6	IYS 20,000 treated oocysts	Yes
	7	IYS 20,000 treated oocysts plus Salinomycin	Yes
	8	IYS 200,000 treated oocysts plus Salinomycin	Yes

In light of the detailed description of the invention and the examples presented above, it can be appreciated that the several aspects of the invention are achieved.

It is to be understood that the present invention has been described in detail by way of illustration and example in order to acquaint others skilled in the art with the invention, its principles, and its practical application. Particular formulations and processes of the present invention are not limited to the descriptions of the specific embodiments presented, but rather the descriptions and examples should be viewed in terms of the claims that follow and their equivalents. While some of the examples and descriptions above include some conclusions about the way the invention may function, the inventors

do not intend to be bound by those conclusions and functions, but put them forth only as possible explanations.

5 It is to be further understood that the specific embodiments of the present invention as set forth are not intended as being exhaustive or limiting of the invention, and that many alternatives, modifications, and variations will be apparent to those skilled in the art in light of the foregoing examples and detailed description. Accordingly, this invention is intended to embrace all such alternatives, modifications, and variations that fall within the spirit and scope of the following claims.

What is claimed is:

1. A preparation for administration by intra yolk sac injection for the prevention and treatment of coccidiosis in members of the class *Aves* comprising live, sporocysts of at least one species of coccidial protozoa and pharmaceutically acceptable carrier, diluent or excipient, the preparation characterized by being substantially
5 free of extraneous bacterial, fungal and viral contaminants.
2. The preparation of claim 1, wherein at least 10% of the total coccidial protozoa in the preparation are sporocysts.
3. The preparation of claim 1, wherein the coccidial protozoa is of the order *Eucoccidiida*.
4. The preparation of claim 1, wherein the genus of the coccidial protozoa is *Eimeria*.
5. The preparation of claim 1, wherein the number of sporocysts per dose of each species of protozoa in the preparation is at least about 50.
6. The preparation of claim 1, wherein the number of sporocysts per dose of each species of protozoa in the preparation is at least about 1,000.
7. The preparation of claim 1, wherein the pharmaceutically acceptable carrier, diluent, or excipient further comprises at least one preservative.
8. The preparation of claim 1, further comprising at least one immune system stimulant.
9. The preparation of claim 1, further comprising at least one additional vaccine preparation for the prevention of a disease of birds.

10. The preparation of claim 1, further comprising at least one growth stimulant.
11. A preparation for administration by intra yolk sac injection for the prevention and treatment of coccidiosis in members of the class *Aves* comprising live, sporulated oocysts of at least one species of coccidial protozoa which have been treated to disrupt the oocyst wall and a pharmaceutically acceptable carrier, diluent or excipient, the preparation characterized by being substantially free of extraneous bacterial, fungal and viral contaminants.
12. The preparation of claim 11, wherein the disruption of the oocyst wall is accomplished by a method chosen from the group consisting of mechanical disruption, chemical disruption and enzymatic disruption.
13. The preparation of claim 11, wherein at least 10% of the total coccidial protozoa in the preparation are sporocysts.
14. The preparation of claim 11, wherein the coccidial protozoa is of the order *Eucoccidiida*.
15. The preparation of claim 11, wherein the genus of the coccidial protozoa is *Eimeria*.
16. The preparation of claim 11, wherein the number of treated oocysts per dose of each species of protozoa in the preparation is at least about 50.
17. The preparation of claim 11, wherein the number of treated oocysts per dose of each species of protozoa in the preparation is at least about 1,000.
18. The preparation of claim 11, wherein the pharmaceutically acceptable carrier, diluent, or excipient further comprises at least one preservative.

19. The preparation of claim 11, further comprising at least one immune system stimulant.
20. The preparation of claim 11, further comprising at least one additional vaccine preparation for the prevention of a disease of birds.
21. The preparation of claim 11, further comprising at least one growth stimulant.
22. A preparation for administration by intra yolk sac injection for the prevention and treatment of coccidiosis in members of the class *Aves* comprising at least about 50 live, sporulated oocysts which have been treated to disrupt the oocyst wall from each of the species of coccidial protozoa *E. acervulina*, *E. maxima* and *E. tenella*
5 and a pharmaceutically acceptable adjuvant, carrier, diluent, or excipient, the preparation characterized by being substantially free of extraneous bacterial, fungal and viral contaminants.
23. The preparation of claim 22, further comprising 30 µg/ml gentamicin.
24. The preparation of claim 22, wherein the coccidial protozoa are unattenuated.
25. A preparation for administration by intra yolk sac injection for the prevention and treatment of coccidiosis in members of the class *Aves* comprising at least about 50 live sporocysts from each of the species of coccidial protozoa *E. acervulina*, *E. maxima* and *E. tenella* and a pharmaceutically acceptable adjuvant, carrier, diluent
5 or excipient, the preparation characterized by being substantially free of extraneous bacterial, fungal and viral contaminants.
26. The preparation of claim 25, further comprising 30 µg/ml gentamicin.
27. The preparation of claim 25, wherein the protozoa are unattenuated.

28. A method for the prevention and treatment of coccidiosis in members of the class *Aves* comprising administration by intra yolk sac injection to newly hatched chicks of a preparation comprising live, sporocysts of at least one species of coccidial protozoa.
29. The method of claim 28, wherein at least 10% of the total coccidial protozoa in the preparation are sporocysts.
30. The method of claim 28, wherein the coccidial protozoa is of the order *Eucoccidiida*.
31. The method of claim 28, wherein the genus of the coccidial protozoa is *Eimeria*.
32. The method of claim 28, wherein the number of sporocysts of each species of coccidial protozoa administered per dose is at least about 50.
33. The method of claim 28, wherein the number of sporocysts of each species of coccidial protozoa administered per dose is at least about 1,000.
34. The method of claim 28, wherein the preparation is administered in conjunction with at least one immune system stimulant.
35. The method of claim 28, wherein the preparation is administered in conjunction with at least one additional vaccine preparation for the prevention of a disease of birds.
36. The method of claim 28, wherein the preparation is administered in conjunction with at least one growth stimulant.
37. The method of claim 28, wherein the preparation is administered in conjunction with at least one anti coccidial drug.

38. A method for the prevention and treatment of coccidiosis in members of the class *Aves* comprising administration by intra yolk sac injection to newly hatched chicks of a preparation comprising live, sporulated oocysts of at least one species of coccidial protozoa which have been treated to disrupt the oocyst wall.
39. The method of claim 38 wherein the disruption of the oocyst wall is accomplished by a method chosen from the group consisting of mechanical disruption, chemical disruption and enzymatic disruption.
40. The method of claim 38, wherein at least 10% of the total coccidial protozoa in the preparation are sporocysts.
41. The method of claim 38, wherein the coccidial protozoa is of the order *Eucoccidiida*.
42. The method of claim 38, wherein the genus of the coccidial protozoa is *Eimeria*.
43. The method of claim 38, wherein the number of treated oocysts of each species of coccidial protozoa administered per dose is at least about 50.
44. The method of claims 38, wherein the number of treated oocysts of each species of coccidial protozoa administered per dose is at least about 1,000.
45. The method of claim 38, wherein the preparation is administered in conjunction with at least one immune system stimulant.
46. The method of claim 38, wherein the preparation is administered in conjunction with at least one additional vaccine preparation for the prevention of a disease of birds.
47. The method of claim 38, wherein the preparation is administered in conjunction with at least one growth stimulant.

48. The method of claim 38, wherein the preparation is administered in conjunction with at least one anti coccidial drug.
49. A method for the prevention and treatment of coccidiosis in members of the class *Aves* comprising administration by intra yolk sac injection to newly hatched chicks of a preparation comprising at least about 50 live, sporulated oocysts of each of the coccidial protozoa *E. acervulina*, *E. maxima* and *E. tenella* which have been
5 treated to disrupt the oocyst wall.
50. The method of claim 49, wherein the coccidial protozoa are unattenuated.
51. The method of claim 49, wherein the treated oocysts are administered within 48 hours of hatching.
52. A method for the prevention and treatment of coccidiosis in members of the class *Aves* comprising administration by intra yolk sac injection to newly hatched chicks of a preparation comprising at least about 50 live, sporocysts of each of the coccidial protozoa *E. acervulina*, *E. maxima* and *E. tenella*.
53. The method of claim 52, wherein the coccidial protozoa are unattenuated.
54. The method of claim 52, wherein the sporocysts are administered within 48 hours of hatching.

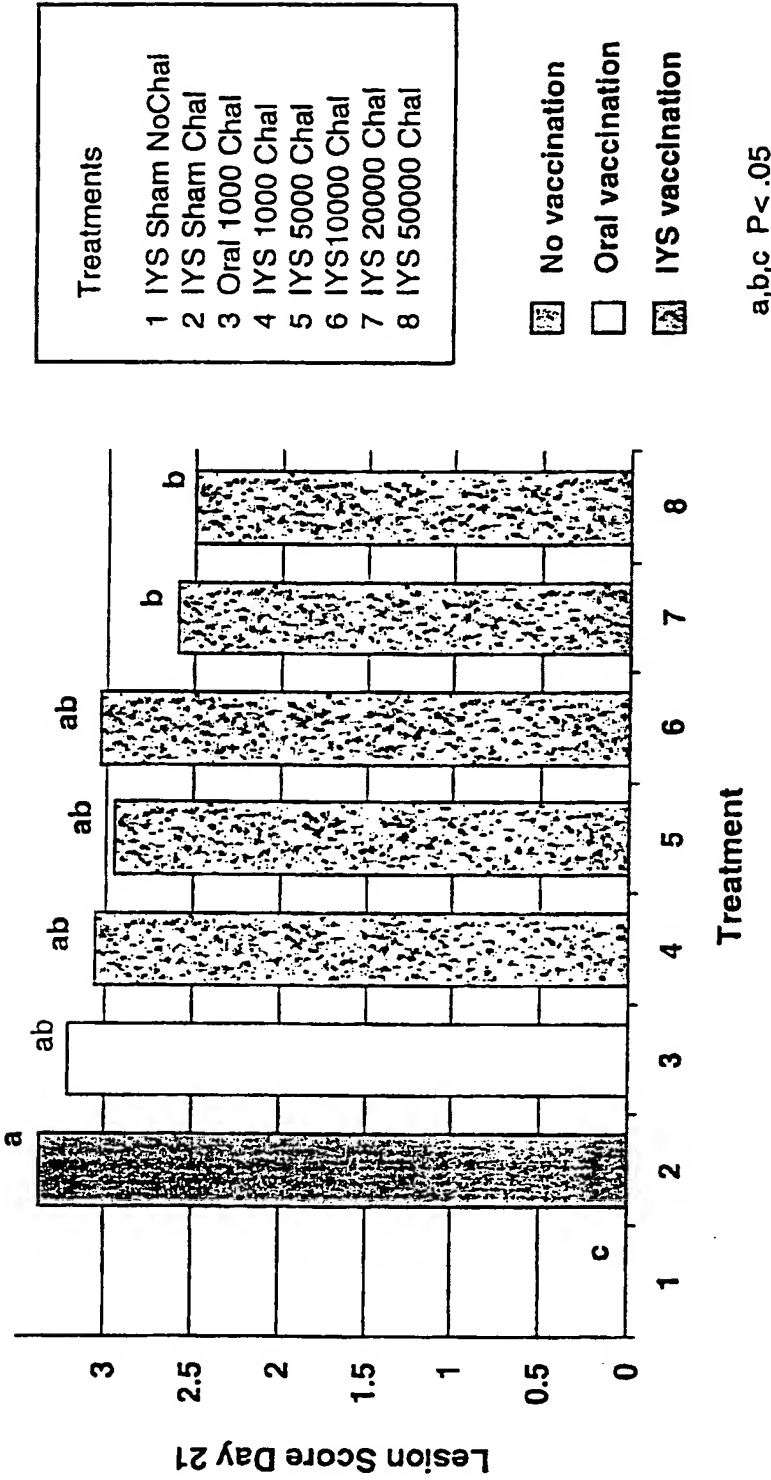


Figure 1

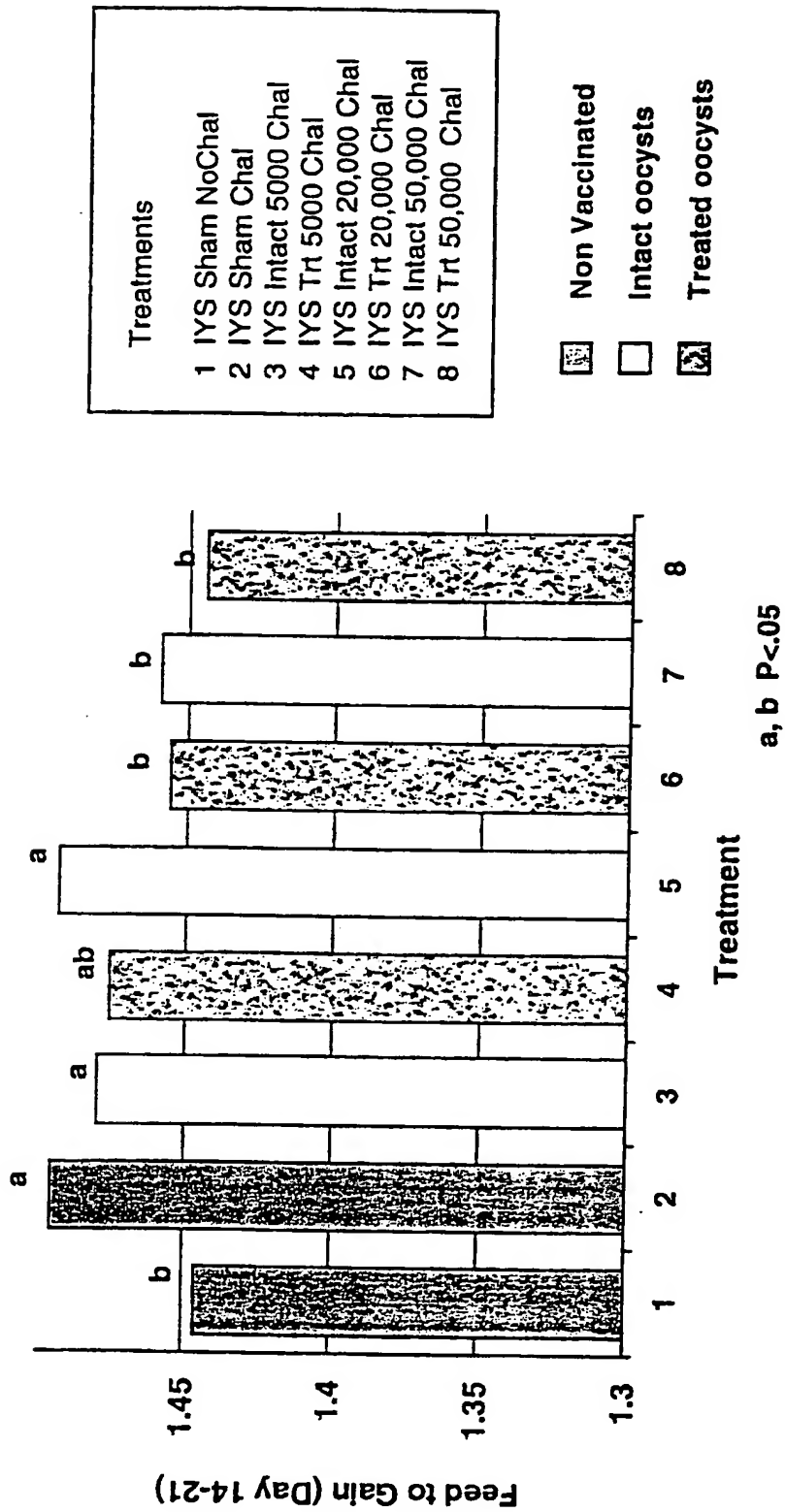


Figure 2

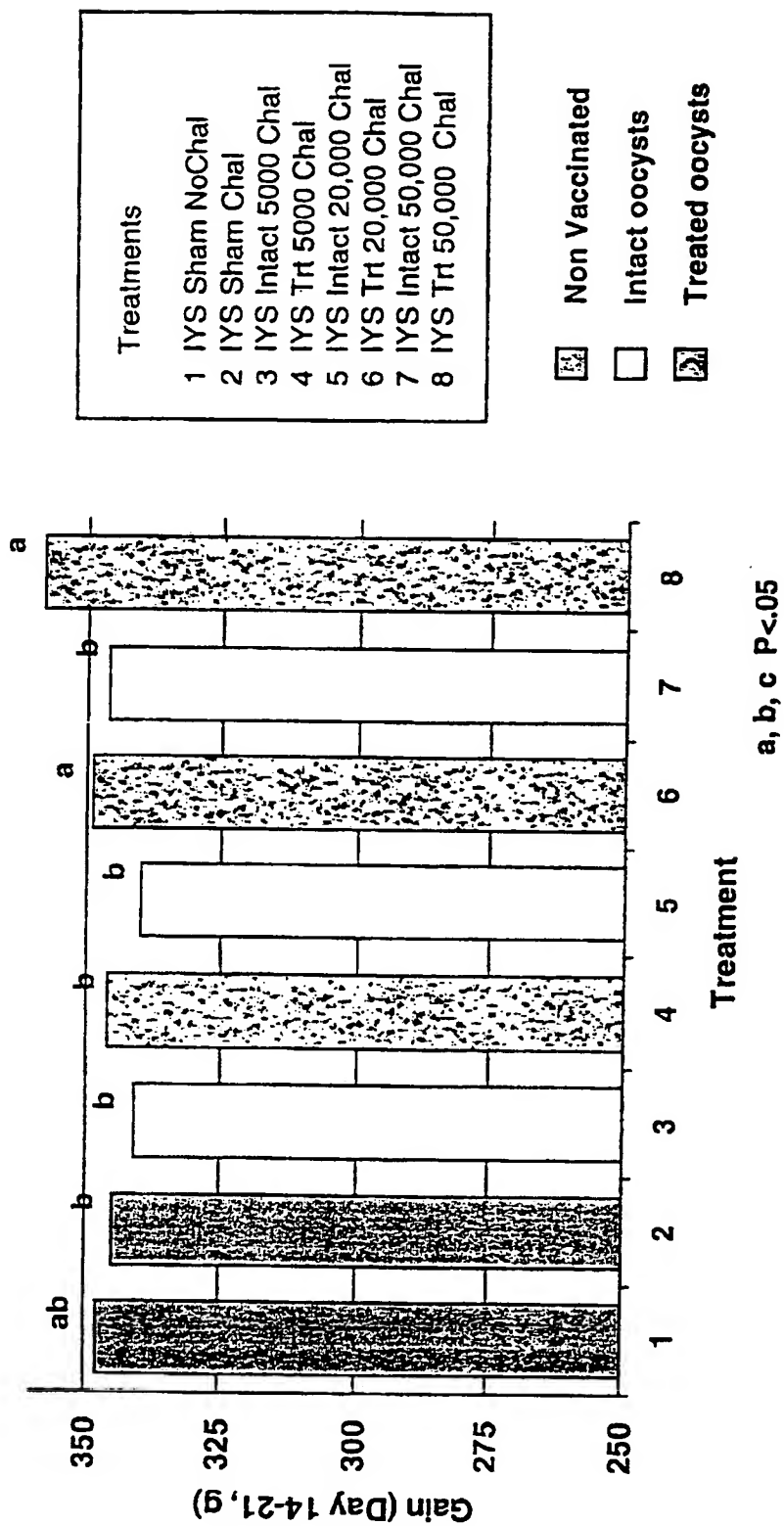


Figure 3

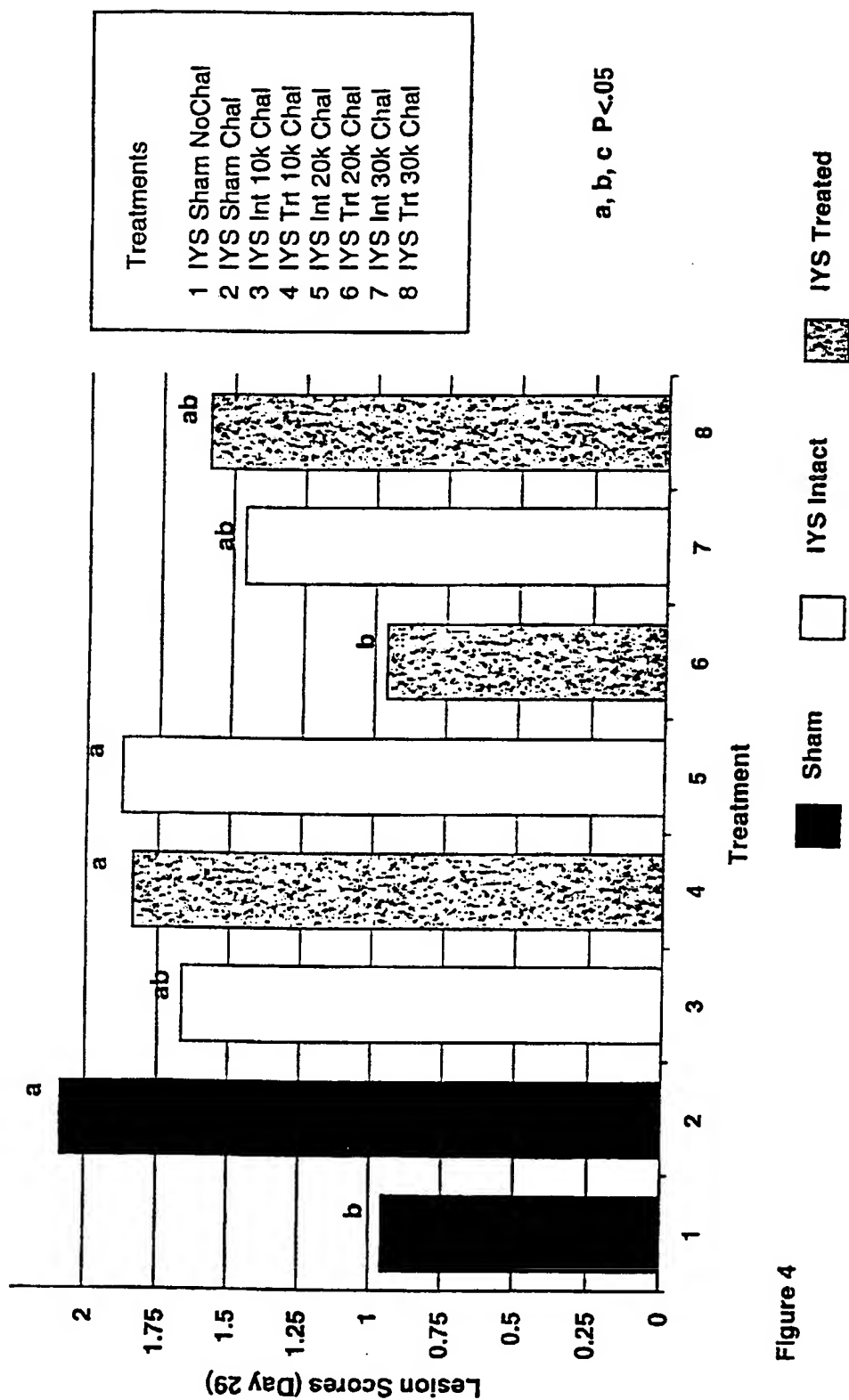


Figure 4

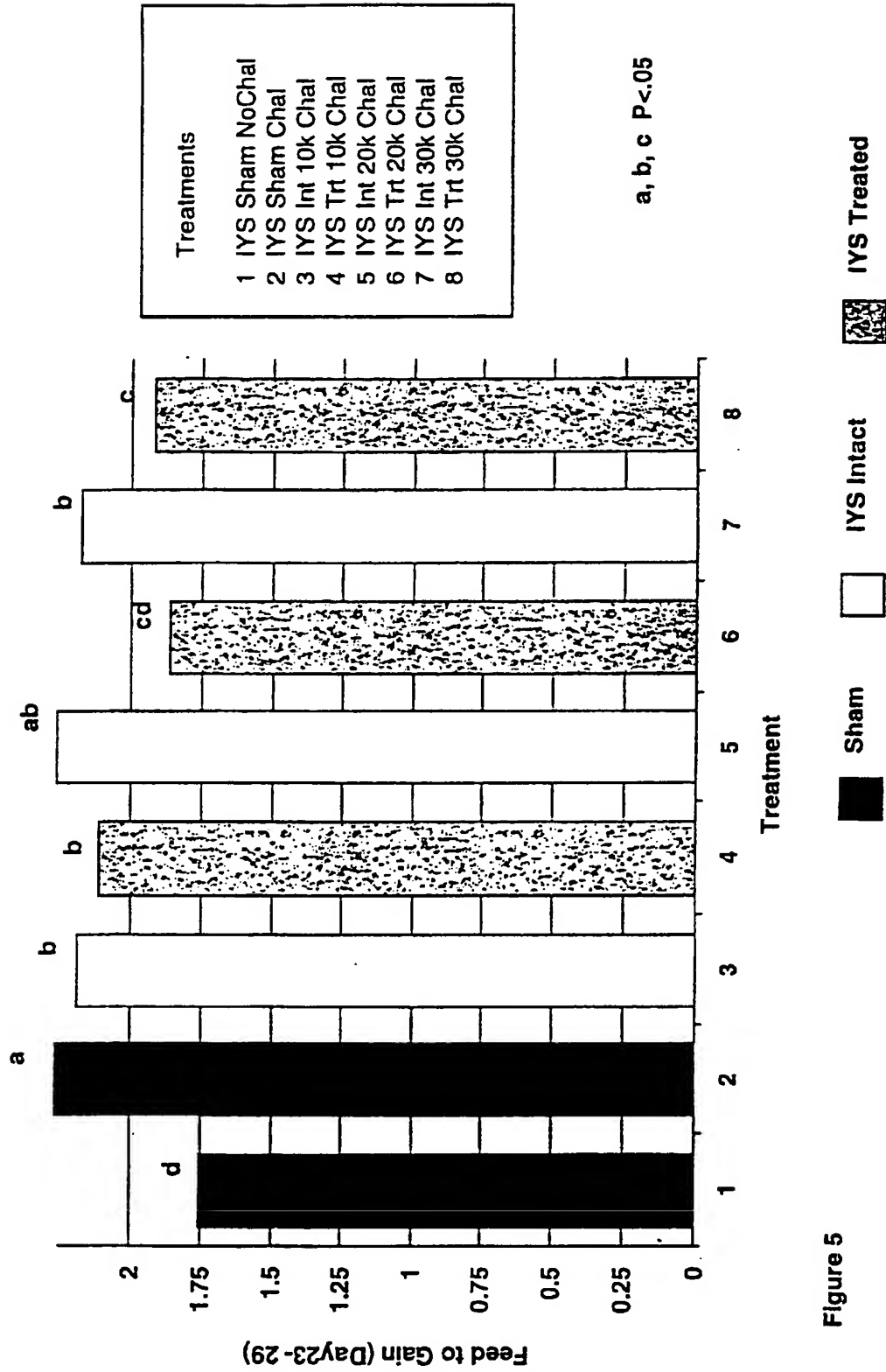


Figure 5

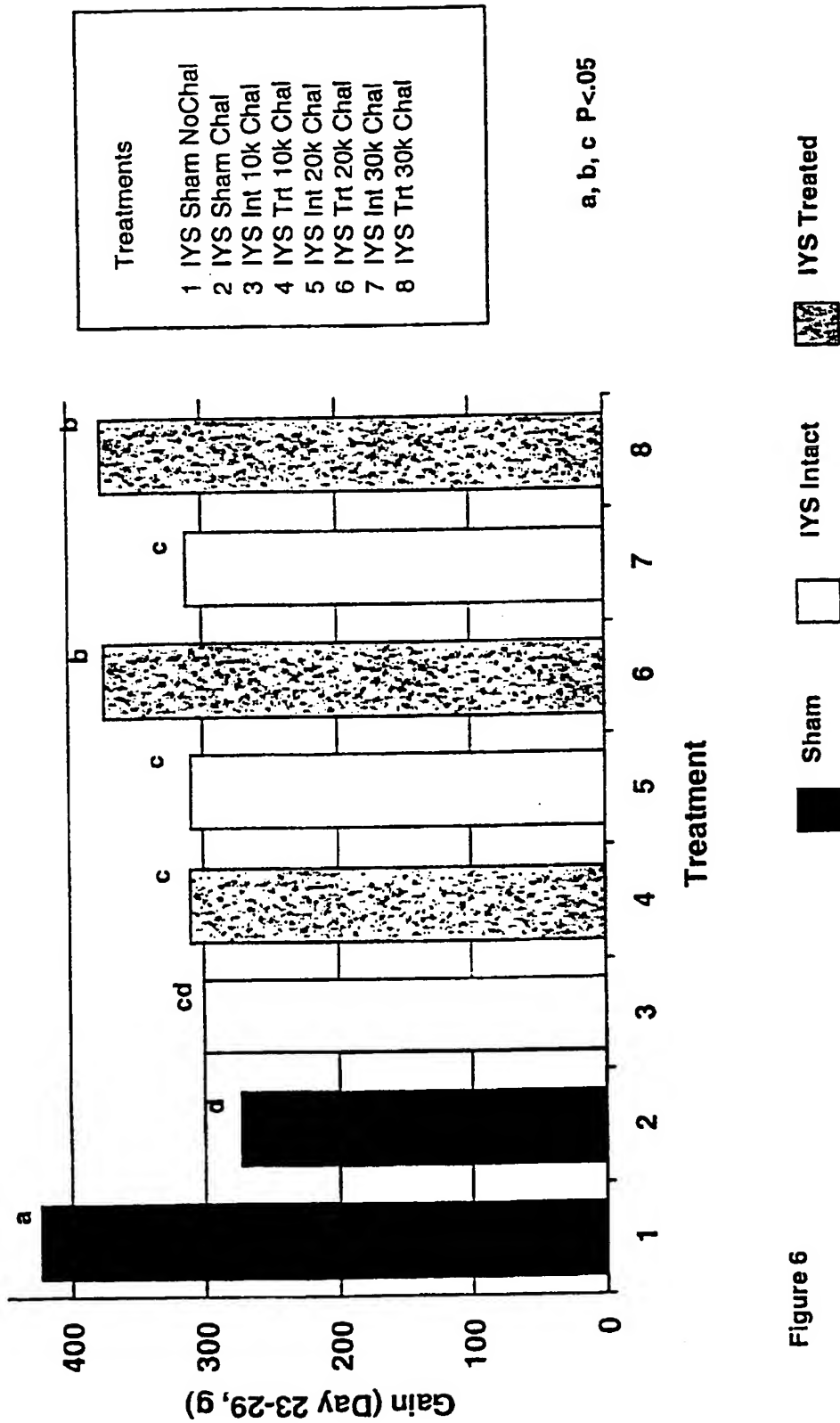


Figure 6

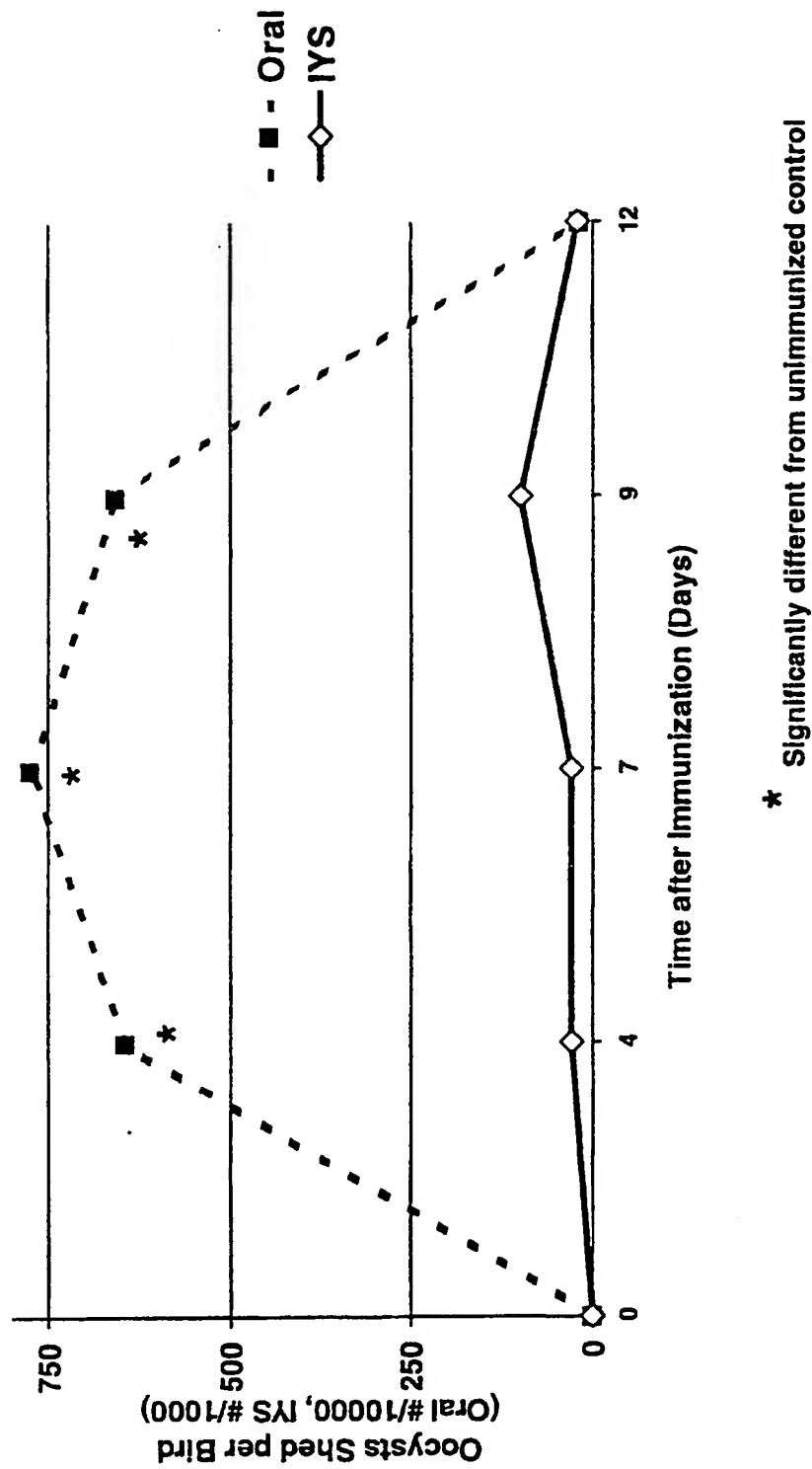


Figure 7

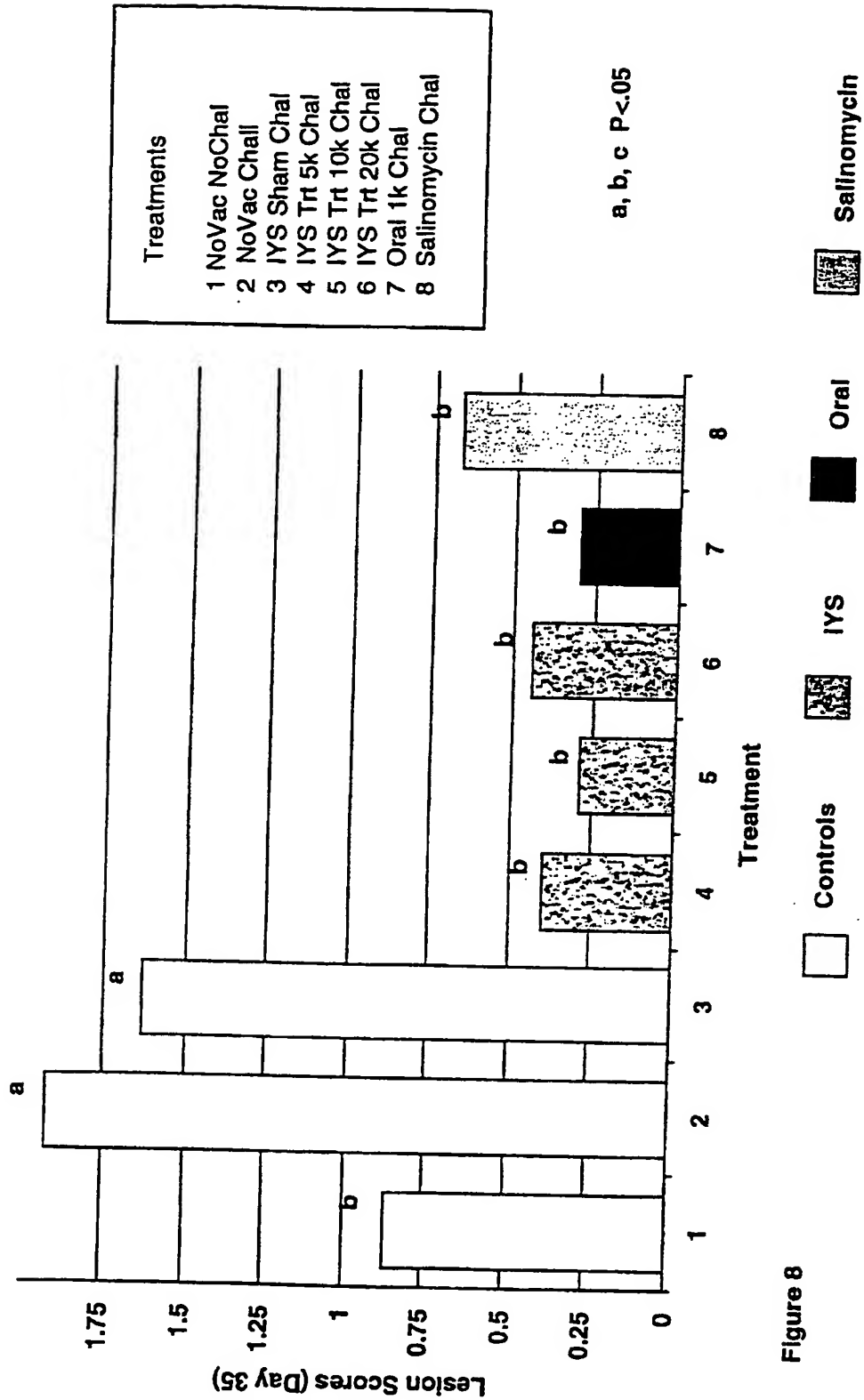


Figure 8

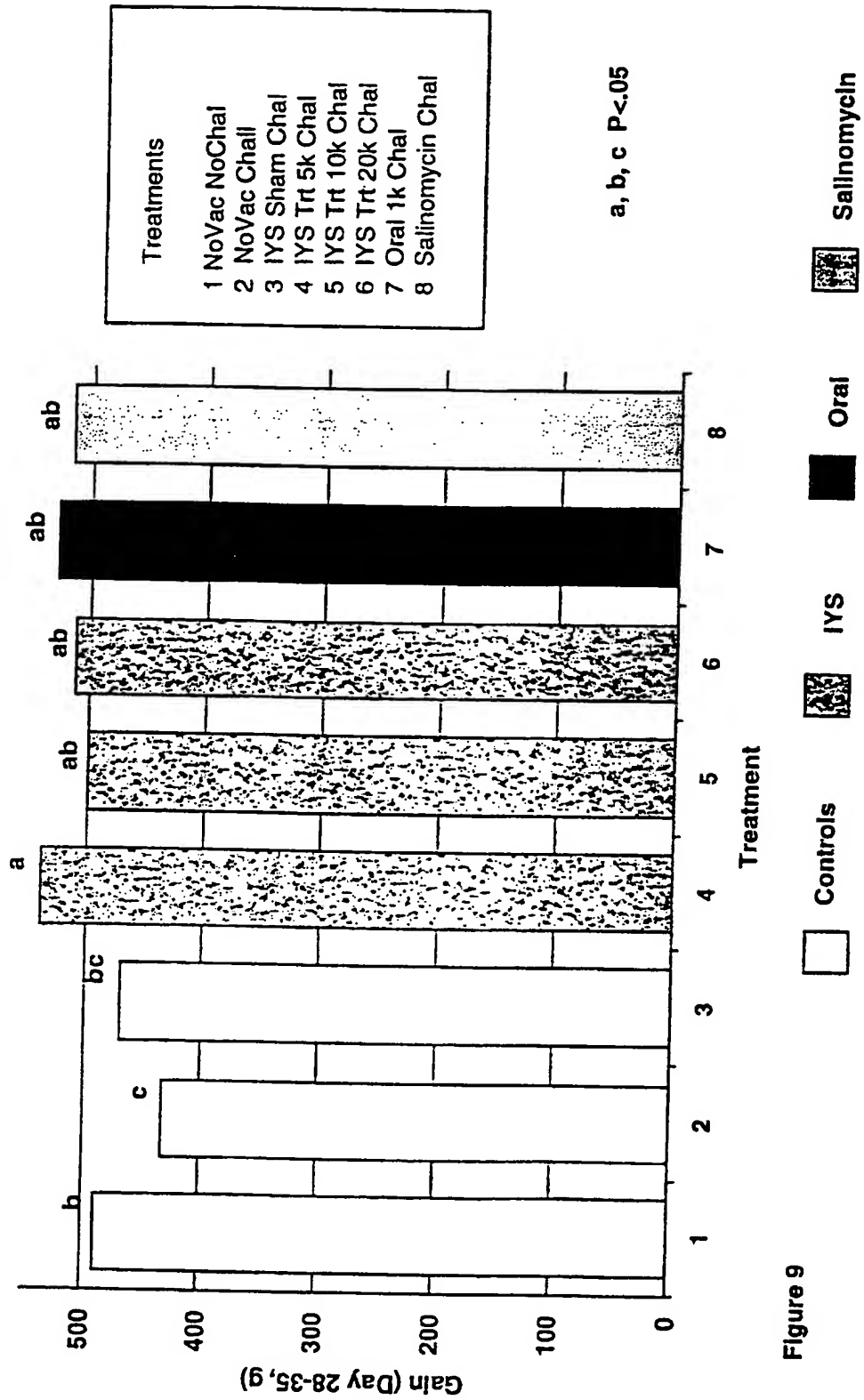


Figure 9

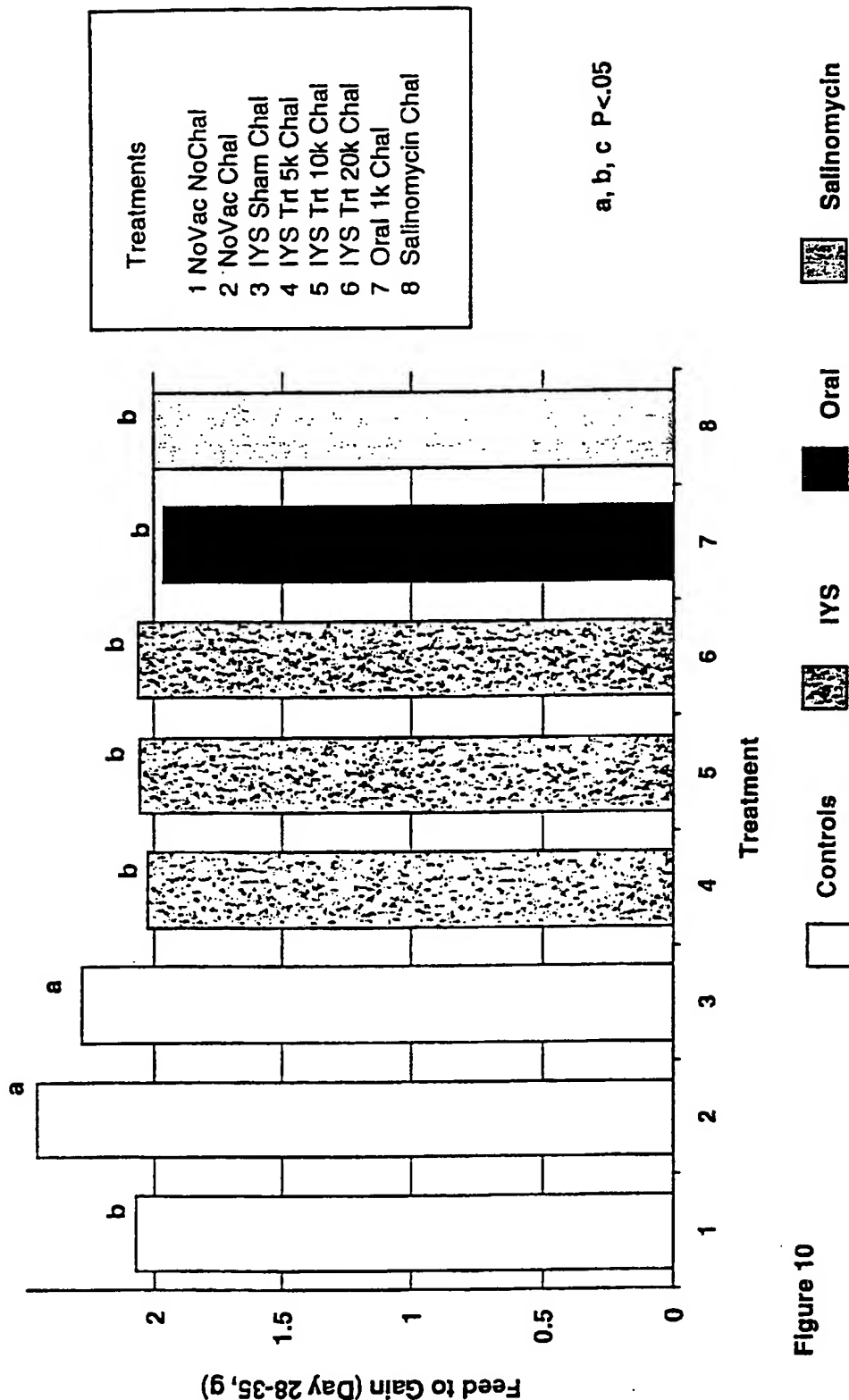
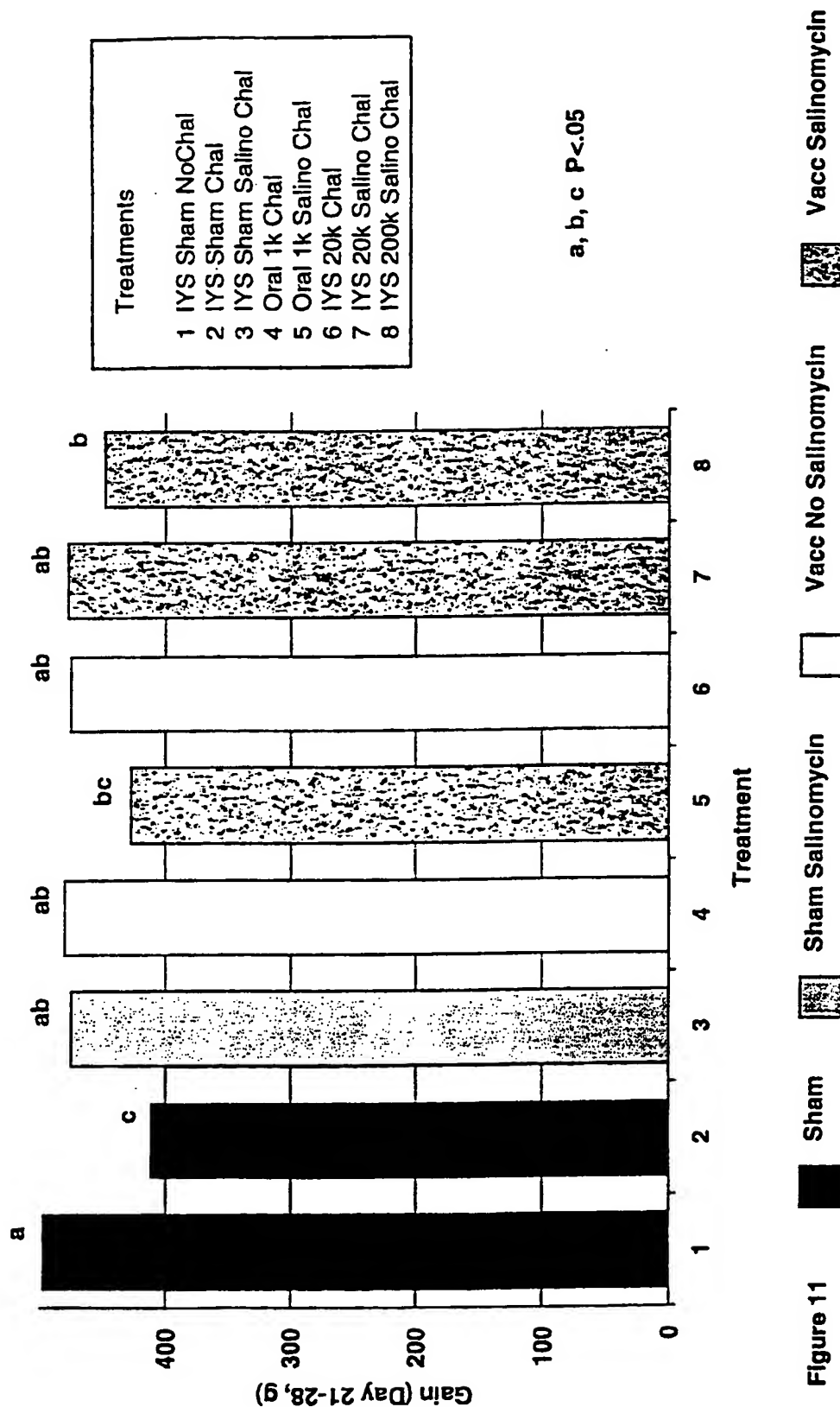


Figure 10



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